

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Donald W. Kufe

Serial No.: 10/733,212

Filed: December 11, 2003

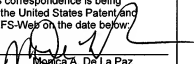
For: REGULATION OF CELL GROWTH BY
MUC1

Confirmation No. 7998

Group Art Unit: 1633

Examiner: Hill, Kevin Kai

Atty. Dkt. No.: GENU:009USD1

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APPEAL BRIEF

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APPEAL BRIEF

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-01450

Dear Sir:

This Appeal Brief is filed in response to the final Office Action mailed on March 2, 2009, and the Advisory Action mailed on May 28, 2009. A Notice of Appeal was filed on July 1, 2009, making this brief due on September 1, 2009. Also included herewith is the fee for the brief. No other fees are believed due in connection with this filing; however, should appellants' payment be missing or deficient, or should any fees be due, the Commissioner is authorized to debit Fulbright & Jaworski L.L.P. Deposit Acct. No. 50-1212/GENU:009USD1/SLH.

I. Real Party in Interest

The real parties in interest are the assignee, the Dana Farber Cancer Institute, Boston, MA, and the licensee, Genus Oncology, LLC.

II. Related Appeals and Interferences

There are no related appeals or interferences.

III. Status of the Claims

Claims 1-56 were filed with the original application. The claims were subjected to a restriction requirement, and as a result, claims 5-10, 16-18, 20-36, 39, 41, 43, 44, 48-51 and 53-56 stand withdrawn. Thus, claims 1-4, 11-15, 19, 37, 38, 40, 42, 45-47 and 52 were examined. Claims 57 was subsequently added, and claims 2-11, 16-18, 20-36, 39, 41, 43, 44, 48-51 and 43-57 were canceled. Thus, claims 1, 12-15, 18, 37, 38, 40, 42, 45-47 and 52 are pending, under examination, stand rejected and are appealed. The pending claims are attached in Appendix A.

IV. Status of the Amendments

The amendments offered following mailing the final Office Action were entered pursuant to the Advisory Action of May 28, 2009.

V. Summary of the Claimed Subject Matter

Independent claim 1 is supported in the specification, for example, at page 1, line 24 to page 2, line 1, and page 17, lines 28-30.

VI. Grounds of Rejection to be Reviewed on Appeal

Are claims 1, 5, 7-9, 13, 15-17 and 22-26 obvious over Li *et al.* (1998; Exhibit 1) in view of Yamamoto *et al.* (1997; Exhibit 2) and Barker *et al.* (U.S. Patent 5, 851,775; Exhibit 3) as evidenced by Zrihan-Licht *et al.* (1994; Exhibit 4) under 35 U.S.C. §103?

VII. Argument

A. Standard of Review

Findings of fact and conclusions of law by the U.S. Patent and Trademark Office must be made in accordance with the Administrative Procedure Act, 5 U.S.C. §706(A), (E), 1994. *Dickinson v. Zurko*, 527 U.S. 150, 158 (1999). Moreover, the Federal Circuit has held that findings of fact by the Board of Patent Appeals and Interferences must be supported by “substantial evidence” within the record. *In re Gartside*, 203 F.3d 1305, 1315 (Fed. Cir. 2000). In *In re Gartside*, the Federal Circuit stated that “the ‘substantial evidence’ standard asks whether a reasonable fact finder could have arrived at the agency’s decision.” *Id.* at 1312. Accordingly, it necessarily follows that an examiner’s position on appeal must be supported by “substantial evidence” within the record in order to be upheld by the Board of Patent Appeals and Interferences.

B. Rejection Under 35 U.S.C. §103

Claims 1, 5, 7-9, 13, 15-17 and 22-26 are rejected as obvious over Li *et al.* (1998; Exhibit 1) in view of Yamamoto *et al.* (1997; Exhibit 2) and Barker *et al.* (U.S. Patent 5, 851,775; Exhibit 3) as evidenced by Zrihan-Licht *et al.* (1994; Exhibit 4). The examiner cites Li and Yamamoto as providing methods of identifying a compound that inhibits binding of the β -catenin tumor progressor to a MUC1 test site. Barker is said to provide motivation for the use of a peptide fragment of β -catenin, and Zrihan-Licht is said to teach that the MUC1 test agent will necessarily be phosphorylated at the YEKV site. Appellant traverses.

i. The Examiner's Burden

In rejecting claims under 35 U.S.C. §103, the examiner bears the initial burden of presenting a *prima facie* case of obviousness. See *In re Rijckaert*, 9 F.3d 1531, 1532, 28 USPQ2d 1955, 1956 (Fed. Cir. 1993). A finding of obviousness requires that “the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains.” 35 U.S.C. §103(a). In setting forth a *prima facie* case of obviousness, it is necessary to show “some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness.” *KSR International Co. v. Teleflex Inc.*, 127 S.Ct. 1727, 82 U.S.P.Q.2d 1385 (2007) (quoting *In re Kahn*, 441 F.3d 977, 988 (Fed. Cir. 2006)).

ii. Appellants' Position

In the present case, there is *no prima facie* case of obviousness for the following reasons. Li teaches that glycogen synthase kinase 3 β binds to an STDRSPYE site in MUC1 and phosphorylates the serine that is adjacent to the proline. This phosphorylation decreases the binding of MUC1 to β -catenin. Li does not teach or suggest that phosphorylation of a YEKV site increases binding of MUC1 to β -catenin. The examiner has cited to FIG. 5 of Li as teaching GSK3 β as the test agent. However, there is no information in Li to teach or suggest that the test agent in Li was phosphorylated at a YEKV site. Nor would this be inherent, as it is possible for a YEKV site to not be phosphorylated, and Li teaches that it is phosphorylation of a serine residue that affects interaction of MUC1 with β -catenin, not YEKV.

Yamamoto does not provide any teaching or suggestion concerning a MUC1 test agent phosphorylated at a YEKV site. Rather, it concerns certain studies demonstrating that DF3

(MUC1) binds directly to β -catenin and that the SXXXXXSSL motif in DF3 is responsible for this interaction.

Further, as admitted by the examiner, neither Li nor Yamamoto teach that the β -catenin test agent is a peptide fragment. Barker is cited as teaching that certain assays may be conducted utilizing a β -catenin fragment that is shorter than the full-length tumor progressor. It is not cited as providing any teaching or suggestion concerning assays concerning any MUC1 test agent, much less one that is phosphorylated at a YEKV site. The examiner admits that neither Li, Yamamoto, nor Barker teach that the MUC1 test agent includes a phosphorylated YEKV site. *See* Final Office Action, page 9.

While Zrihan-Licht discloses that MUC1 proteins are “extensively phosphorylated” and that phosphorylation occurs “primarily on tyrosine residues” (Abstract), it does not specifically teach phosphorylation of the YEKV site of MUC1. Indeed, the MUC1 protein includes 13 tyrosine residues, of which 7 are in the cytoplasmic domain, and there is no information in this reference or in any of the other references to suggest that the YEKV tyrosine residue, out of all of the amino acids of MUC1, is critical for binding to β -catenin. Further, Zrihan-Licht teaches that other residues may undergo phosphorylation, including serine residues. *See* p. 131, right col., third para. Still further, Zrihan-Licht teaches that the sequence YEEV is important for interaction with SH2 domain-containing tyrosine kinases, thus teaching away from the importance of a YEKV site. In addition, one of ordinary skill in the art would further be led away from the importance of phosphorylation of a YEKV site because, as discussed above, Li teaches that it is a serine residue that affects interaction of MUC1 with β -catenin and Yamamoto teaches that the SXXXXXSSL motif in DF3 is responsible for this interaction.

Thus, it is again submitted that there is no *prima facie* case of obviousness based on the combination of references cited by the examiner. There is no rationale that would have led one of ordinary skill in the art, at the time of the invention, to believe that the YEKV site of MUC1 is critical for binding to β -catenin, and thus a critical target for screening.

iii. **The Examiner's Rebuttal Fails**

In the Advisory Action mailed on May 28, 2009, the examiner found the preceding line of argument unpersuasive, and offered the following points in rebuttal.

First, it was argued that appellants were improperly addressing the references individually, and not as a whole. This is incorrect. Appellants were pointing out specific defects in the references, and the incorrect nature of the examiner's assumptions therefrom. When viewed in light of these critical deficiencies, the references cannot, even when taken as a whole, suggest the present invention. This is because they neither individually *nor collectively* provide any evidence that the YEKV motif is integral to β -catenin's interaction with MUC1.

Second, turning to Li, the examiner argues that the reference teaches that tyrosine residues flank the identified β -catenin binding motif, and that modification of a serine residue near a YEKV tyrosine did not eliminate interaction with β -catenin. From this, the examiner finds that "Li neither teaches away, discredits or otherwise discourage[s] the ordinary artisan from determining the role tyrosine phosphorylation may play in the interaction between MUC-1 and β -catenin." This very statement highlights the improper nature of the rejection. The claimed invention is not a method of determining *whether* tyrosines generally play a role, but assessing the effects of compounds on this action *after* it was determined that a specific tyrosine *does* play a role.

Third, the examiner makes a similar misapplication of the teachings of Yamamoto. As acknowledged, Yamamoto acknowledged that “it is not known if tyrosine sites influence binding of catenins to the serine rich motif.” A more equivocal statement can hardly be imagined. Yet somehow, the examiner contorts this quote to into a “suggest[ion that] the phosphorylation of one or more of the seven tyrosine residues in the MUC1 cytoplasmic domain ... [is a] possible regulatory feature, wherein the YEKV site is immediately adjacent to the serine rich motif.” To call this statement rank speculation would be too kind – it is nothing short of an outright misrepresentation of the teachings of the reference, as the previous quote from Yamamoto clearly disavows any evidence that tyrosines, much less YEKV tyrosines, are involved. The examiner, knowing this, hedges his bet by stating that “those of ordinary skill in the art were motivated to determine if other phosphorylated residues in the MUC1 cytoplasmic domain were responsible for the interaction between MUC1 and β -catenin.” Again, appellants are *not* claiming to “determine” whether phosphorylated MUC1 residues have an impact on function, which this language would imply. Instead, they are claiming to exploit the finding, made by the inventors and *not* by Li or Yamamoto, that YEKV *is* in fact critical to MUC1’s interaction with β -catenin. Without this knowledge, the prior art at best the art leaves one to pursue a general line of research that may or may not lead to fruition. This does not qualify as obvious. *In re O’Farrell*, 853 F.2d 894, 903, 7 USPQ2d 1673, 1681 (Fed. Cir. 1988).

Fourth, the examiner argues that appellants have overlooked the “emphasis” Zrihan “postulated” that MUC1 tyrosines interact with SH2 domain-containing proteins, while admitting that YEEV motifs are preferred. Thus, the examiner argues that “it does not teach away from all other tyrosines.” Whether or not this is true, it highlights the fact that Zrihan certainly does not *suggest* the significance of YEKV motifs, and *that* is what is being claimed

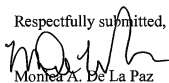
here. Thus, this reference too lacks any reasonable teaching or inference that would guide the skilled artisan to YEKV. At most, this is an invitation to invent, and it certainly cannot obviate appellants' invention based on that alone.

In conclusion, appellants submit that the following summation, offered by the examiner, highlights the baseless nature of the rejection: "The tyrosine phosphorylation of MUC1, and the YEKV site in particular, *necessarily flows* from the signal transduction pathways in cancer cells of Li *et al.* (1998), Yamamoto *et al.* and Barker." This language smacks of a inherency theory, which has no basis in an obviousness rejection. The examiner is simply grasping at straws in an vain effort to support a rejection that lacks the required teaching, suggestion and motivation in the cited art. In the end, one of ordinary skill would have no reasonable expectation of success that phosphorylation of a YEKV site would be important for interaction with β -catenin. Therefore, the Examiner has not set forth a *prima facie* case of obviousness.

C. Conclusion

In light of the foregoing, appellants respectfully submit that all pending claims are non-obvious under 35 U.S.C. §103. Therefore, it is respectfully requested that the Board reverse the pending rejection.

Respectfully submitted,



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Date: August 25, 2009

VIII. APPENDIX A – APPEALED CLAIMS

1. A method of identifying a compound that inhibits binding of MUC1 to a tumor progressor, the method comprising:

(a) providing a MUC1 test agent, wherein the MUC1 test agent comprises a phosphorylated YEKV site;

(b) providing a tumor progressor test agent that binds to the phosphorylated MUC1 test agent;

(c) contacting the phosphorylated MUC1 test agent with the tumor progressor test agent in the presence of a test compound; and

(d) determining whether the test compound inhibits binding of the phosphorylated MUC1 test agent to the tumor progressor test agent.

5. The method of claim 1, wherein the tumor progressor test agent is a β -catenin test agent.

7. The method of claim 1, wherein the contacting is carried out in a cell-free system.

8. The method of claim 1, wherein the contacting occurs in a cell.

9. The method of claim 1, wherein the test compound is a peptide fragment of the tumor progressor.

13. The method of claim 9, wherein the tumor progressor test agent is a β -catenin test agent.

15. The method of claim 9, wherein the contacting is carried out in a cell-free system.

16. The method of claim 9, wherein the contacting occurs in a cell.

17. The method of claim 1, wherein the MUC1 test agent comprises SEQ ID NO:1.

22. The method of claim 8, wherein the cell is a cancer cell.
23. The method of claim 22, wherein the cancer cell expresses MUC1.
24. The method of claim 22, wherein the cancer cell is a breast cancer cell, a lung cancer cell, a colon cancer cell, a pancreatic cancer cell, a renal cancer cell, a stomach cancer cell, a liver cancer cell, a bone cancer cell, a hematological cancer cell, a neural tissue cancer cell, a melanoma cell, an ovarian cancer cell, a testicular cancer cell, a prostate cancer cell, a cervical cancer cell, a vaginal cancer cell, or a bladder cancer cell.
25. The method of claim 5, wherein providing a phosphorylated MUC1 test agent comprises combining a MUC1 test agent, a tumor progressor test agent with kinase activity, and ATP, wherein a MUC1 test agent phosphorylated at a YEKV site is formed.
26. The method of claim 25, wherein the tumor progressor test agent with kinase activity is c-src, EGF-R, or PKC δ .

IX. APPENDIX B – EVIDENCE CITED

Exhibit 1 – Li *et al.* (1998)

Exhibit 2 – Yamamoto *et al.* (1997)

Exhibit 3 – Barker *et al.* (U.S. Patent 5,851,775)

Exhibit 4 – Zrihan-Licht *et al.* (1994)

X. APPENDIX C – RELATED PROCEEDINGS

None

The Epidermal Growth Factor Receptor Regulates Interaction of the Human DF3/MUC1 Carcinoma Antigen with c-Src and β -Catenin*

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The DF3/MUC1 mucin-like, transmembrane glycoprotein is aberrantly overexpressed in most human carcinomas. The MUC1 cytoplasmic domain interacts with the c-Src tyrosine kinase and thereby increases binding of MUC1 and β -catenin. In the present work, coimmunoprecipitation studies demonstrate that MUC1 associates constitutively with the epidermal growth factor receptor (EGF-R) in human ZR-75-1 breast carcinoma cells. Immunofluorescence studies show that EGF-R and MUC1 associate at the cell membrane. We also show that the activated EGF-R phosphorylates the MUC1 cytoplasmic tail on tyrosine at a YEKV motif that functions as a binding site for the c-Src SH2 domain. The results demonstrate that EGF-R-mediated phosphorylation of MUC1 induces binding of MUC1 to c-Src in cells. Moreover, *in vitro* and *in vivo* studies demonstrate that EGF-R increases binding of MUC1 and β -catenin. These findings support a novel role for EGF-R in regulating interactions of MUC1 with c-Src and β -catenin.

The epidermal growth factor receptor (EGF-R, HER1)¹ is a member of a family of transmembrane receptor tyrosine kinases that includes HER2/neu, HER3, and HER4 (1). EGF-R is activated by EGF, transforming growth factor- α , amphiregulin, and betacellulin. Following ligand binding, inactive monomeric EGF-R undergoes homodimerization or heterodimerization

with other members of the HER family (2). Activation of EGF-R is associated with phosphorylation of specific tyrosine residues in the cytoplasmic region and thereby the recruitment of effector proteins that contain SH2 domains. For example, interaction of EGF-R with the Shc and Grb2 adaptor proteins links receptor activation to the Ras signaling pathway (3–5). Activation of EGF-R is also associated with the formation of complexes with the c-Src nonreceptor tyrosine kinase (6, 7). The finding that overexpression of EGF-R in fibroblasts confers growth in soft agar and induces tumorigenicity in nude mice has indicated that EGF-R can function as an oncogene (8, 9). Other studies in cells overexpressing both EGF-R and c-Src have shown that c-Src potentiates EGF-R-mediated tumorigenesis (7). The interaction between EGF-R and c-Src is further supported by the demonstration that c-Src is required for EGF-R-dependent mitogenesis (10).

The human DF3/MUC1 mucin-like glycoprotein is highly overexpressed by human carcinomas (11). Whereas MUC1 expression is restricted to the apical borders of normal secretory epithelium, this transmembrane protein is aberrantly expressed by carcinoma cells at high levels over the entire cell surface (11). The MUC1 protein consists of an N-terminal ectodomain with variable numbers of conserved 20 amino acid tandem repeats that are subject to O-glycosylation (12, 13). The C-terminal region includes a transmembrane domain and a 72-amino acid cytoplasmic tail that contains seven sites for tyrosine phosphorylation. The >250-kDa ectodomain associates with the ~25 kDa C-terminal region as a heterodimer at the cell surface. β -Catenin, a component of the adherens junction of mammalian epithelial cells, binds directly to MUC1 at a SAGNGSSL motif in the cytoplasmic domain (14). Similar motifs are responsible for interactions of β -catenin with E-cadherin and the adenomatous polyposis coli tumor suppressor (15–17). Glycogen synthase kinase 3 β (GSK3 β) also binds to MUC1 and phosphorylates serine in a SPYEKV site adjacent to that for the β -catenin interaction (18). More recent studies have shown that c-Src phosphorylates the SPYEKV site on tyrosine (19). The findings also demonstrate that c-Src increases, while GSK3 β down-regulates, the interaction between MUC1 and β -catenin (18, 19).

The present studies demonstrate that the EGF-R interacts with MUC1. The activated EGF-R phosphorylates MUC1 on the YEKV motif in the cytoplasmic tail. The results also demonstrate that EGF-R-mediated phosphorylation of MUC1 induces binding of MUC1 to c-Src and β -catenin.

MATERIALS AND METHODS

Cell Culture—Human ZR-75-1 carcinoma cells were grown in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (HI-FBS), 100 units/ml penicillin, 100 μ g/ml streptomycin, and 2 mM L-glutamine. HCT116 and 293 cells were cultured in Dulbecco's modified Eagle's medium with 10% HI-FBS, 100 units/ml penicillin and 100 μ g/ml streptomycin. In certain studies, cells were cultured in medium with 0.1% HI-FBS for 24 h and then stimulated with 10 ng/ml EGF (Calbiochem-Novabiochem, San Diego, CA) for 5 min at 37 °C.

Cell Transfections—Wild-type MUC1 containing 40 tandem repeats was excised from pCMV-MUC1 (19) by *Nde*I and *Eco*RI digestion and integrated into the *Nde*I/*Eco*RI site of the mammalian expression vector pIRESpuro2 (CLONTECH, Palo Alto, CA). The pIRESpuro2-MUC1 (Y46F) mutant vector was constructed by insertion of the 3'-terminal region from pCMV-MUC1(Y46F) (19) into pIRESpuro2-MUC1 deleted at the 3'-terminal region of MUC1 by Bsu36I. 293 cells were transiently transfected with pcDNA3.1/EGF-R and/or pIRESpuro2-MUC1 by Lipo-

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¹ The abbreviations used are: EGF, epidermal growth factor; EGF-R, EGF receptor; GSK3 β , glycogen synthase kinase 3 β ; CD, cytoplasmic domain; HI-FBS, heat-inactivated fetal bovine serum; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase.

fectAMINE (Life Technologies Inc.). Cell lysates were prepared at 48 h after transfection, pIRESpu2, pIRESpu2-MUC1, and pIRESpu2-MUC1(Y46F) were transfected into HCT116 colon carcinoma cells by LipofectAMINE. Stable transfectants were selected in the presence of 0.4 μ g/ml of puromycin (Calbiochem-Novabiochem Co., San Diego, CA).

Lysate Preparation—Lysates from subconfluent cells were prepared as described previously (18).

Immunoprecipitation and Immunoblotting—Equal amounts of protein from the cell lysates were incubated with mouse or rabbit IgG, monoclonal antibody DF3 (anti-MUC1) (11), or anti-EGF-R (Santa Cruz Biotechnology, Santa Cruz, CA). The immune complexes were prepared

as described previously (18), separated by SDS-PAGE, and transferred to nitrocellulose membranes. The immunoblots were probed with monoclonal antibody DF3, anti-EGF-R, anti-c-Src (Upstate Biotechnology, Lake Placid, NY), anti-P-Tyr (RC20H; Transduction Laboratories, San Diego, CA), or anti- β -catenin (Zymed Laboratories Inc., San Francisco, CA). Reactivity was detected with horseradish peroxidase-conjugated second antibodies and chemiluminescence (PerkinElmer Life Sciences).

Immunofluorescence Microscopy—ZR-75-1 cells were fixed with 4% paraformaldehyde for 10 min at room temperature and blocked with 5% fatty acid-free BSA (Sigma) and 5% normal goat serum (Jackson ImmunoResearch Laboratories Inc., Westgrove, PA) in phosphate-buffered saline (blocking buffer) for 45 min at room temperature. After incubation with anti-MUC1 (1:400) and rabbit anti-EGF-R (1:100) in blocking buffer for 14 h at 4 °C, the cells were washed with phosphate-buffered saline and incubated with fluorescein-conjugated anti-rabbit IgG (1:100) or Texas Red-conjugated anti-mouse IgG (1:200) (Jackson ImmunoResearch Laboratories Inc., Westgrove, PA) for 45 min at room temperature. The cells were then mounted onto coverslips using the slow fade mounting kit (Molecular Probes, Eugene, OR) and analyzed by confocal microscopy (inverted Zeiss LSM 510). Images were captured at 0.6-nm increments along the z axis under $\times 63$ magnification and converted to composite images by ImageSpace 3.10 software (Molecular Dynamics, Sunnyvale, CA).

In Vitro Phosphorylation—Purified wild-type and mutant MUC1/CD proteins were incubated with 0.1 unit of purified EGF-R (Calbiochem-Novabiochem Co., San Diego, CA) in 20 μ l of kinase buffer (20 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 5 mM dithiothreitol). Kinase reactions and analysis of the reaction products were performed as described previously (19).

Binding Studies—Purified wild-type and mutant MUC1/CD proteins were incubated with 0.1 unit of EGF-R in the absence and presence of 200 μ M ATP for 30 min at 30 °C. GST-c-Src or GST- β -catenin bound to glutathione beads was then added, and the reaction was incubated for 1 h at 4 °C. After washing, the precipitated proteins were subjected to immunoblot analysis with anti-MUC1/CD (18) or anti-Tyr(P).

RESULTS AND DISCUSSION

MUC1 Associates with EGF-R—To determine whether MUC1 forms a complex with EGF-R, anti-MUC1 immunoprecipitates from lysates of human ZR-75-1 cells were analyzed by immunoblotting with anti-EGF-R. The results demonstrate that EGF-R coprecipitates with MUC1 (Fig. 1A). As a control, there was no detectable EGF-R in immunoprecipitates prepared with IgG (Fig. 1A). In the reciprocal experiment, analysis of anti-EGF-R immunoprecipitates with anti-MUC1 confirmed that EGF-R associates with MUC1 (Fig. 1B). To extend these findings, 293 cells, which express low levels of EGF-R and are negative for MUC1 (18), were transfected to express EGF-R and MUC1. Immunoblot analysis of anti-MUC1 immunoprecipitates with anti-EGF-R demonstrated coprecipitation of

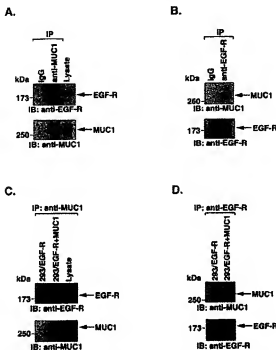


FIG. 1. Interaction of MUC1 with EGF-R. **A** and **B**, lysates from ZR-75-1 cells were subjected to immunoprecipitation (IP) with anti-MUC1 (**A**) or anti-EGF-R (**B**). Rabbit or mouse IgG was used as a control. The immunoprecipitates were analyzed by immunoblotting (IB) with anti-EGF-R and anti-MUC1. **C** and **D**, 293 cells were transiently transfected with EGF-R or EGF-R + MUC1. At 48 h after transfection, the cells were harvested, and lysates were subjected to immunoprecipitation with anti-MUC1 (**C**) or anti-EGF-R (**D**). The immunoprecipitates were analyzed by immunoblotting with anti-EGF-R and anti-MUC1.

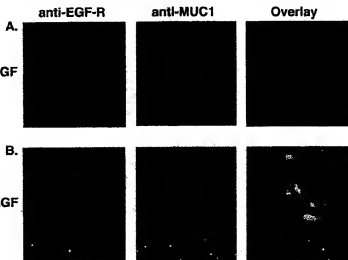


FIG. 2. Colocalization of EGF-R and MUC1 in ZR-75-1 cells. ZR-75-1 cells were grown to 80% confluence in a Lab-Tek II Chamber Slide and then incubated in medium with 0.1% serum for 24 h. The cells were treated without (**A**) or with (**B**) 10 ng/ml EGF for 5 min, fixed, and double-stained with anti-EGF-R (green) and anti-MUC1 (red).

EGF-R and MUC1 (Fig. 1C). Similar results were obtained when anti-EGF-R immunoprecipitates were analyzed by immunoblotting with anti-MUC1 (Fig. 1D). These findings demonstrate that MUC1 constitutively associates with EGF-R.

Colocalization of EGF-R and MUC1 by Immunofluorescence Microscopy—To assess the subcellular localization of MUC1 and EGF-R, confocal microscopy was performed with rabbit anti-EGF-R and mouse anti-MUC1 antibodies. In control ZR-75-1 cells, EGF-R was distributed uniformly over the cell membrane (Fig. 2A, left). Similar findings were obtained for the distribution of MUC1 (Fig. 2A, middle). Overlay of the EGF-R (green) and MUC1 (red) signals supported colocalization (red + green → yellow) (Fig. 2A, right). Following EGF stimulation, the EGF-R signals were clustered in patches at the cell membrane (Fig. 2B, left). An identical pattern was observed for MUC1 (Fig. 2B, middle). Moreover, overlay of the signals showed that EGF-R and MUC1 colocalize in clusters at the cell membrane (Fig. 2B, right). Analysis of the control and EGF-stimulated cells by immunoprecipitation studies demonstrated no detectable difference in the association between EGF-R and MUC1 (data not shown). These findings and those obtained in coprecipitation studies demonstrate that MUC1 and EGF-R associate constitutively at the cell membrane.

EGF-R Phosphorylates MUC1 *In Vitro* and *In Vivo*—To determine whether EGF-R phosphorylates MUC1, anti-MUC1 immunoprecipitates from control and EGF-stimulated ZR-75-1 cells were analyzed by immunoblotting with anti-Tyr(P). The results demonstrate a detectable level of tyrosine-phosphorylated MUC1 in control cells (Fig. 3A). Moreover, EGF stimulation was associated with an increase in phosphorylation of MUC1 on tyrosine (Fig. 3A). EGF-induced tyrosine phosphorylation of MUC1 was also observed in 293 cells transfected to express EGF-R and MUC1 (Fig. 3B). The 72-amino acid MUC1 cytoplasmic domain (MUC1/CD) contains 7 tyrosines (see schema in Fig. 4D). To define potential sites of EGF-R phosphorylation, we incubated the MUC1 cytoplasmic domain (MUC1/CD) with EGF-R and [γ -³²P]ATP. Analysis of the reaction products demonstrated that EGF-R phosphorylates MUC1/CD (Fig. 3C). Mutation of the Tyr³⁵ site to Phe had no detectable effect on EGF-R-mediated phosphorylation of MUC1/CD (Fig. 3C). There was also no apparent effect when the Tyr²⁵⁰ or Tyr²⁵⁸ sites were mutated to Phe (Fig. 3C). By contrast, incubation of MUC1/CD(Y46F) with EGF-R was associated with a marked decrease in phosphorylation as compared with that found with wild-type MUC1/CD (Fig. 3C). Mutation of Tyr²⁵⁸ also resulted in decreased phosphorylation, but to a lesser extent than that obtained with Y46F (Fig. 3C). To determine whether the Tyr⁴⁶ site is phosphorylated *in vivo*, human HCT116 cells, which express EGF-R and not MUC1, were stably transfected to express the empty vector, wild-type MUC1, or the MUC1(Y46F) mutant. Analysis of anti-MUC1 immunoprecipitates with anti-Tyr(P) demonstrated that EGF-mediated phosphorylation of MUC1(Y46F) is decreased compared with that obtained with wild-type MUC1 (Fig. 3D). Relative intensities of the anti-Tyr(P) signals were determined by densitometric scanning (Fig. 3D). Similar results were obtained in three separate experiments (legend to Fig. 3D). The findings that the MUC1(Y46F) mutation decreases tyrosine phosphorylation only in part is in concert with additional tyrosine sites in the MUC1/CD, which can function as substrates for other tyrosine kinases. These results thus demonstrate that EGF-R phosphorylates MUC1 on Tyr⁴⁶ *in vitro* and in cells.

EGF-R Regulates Interaction of MUC1 with c-Src and β -Catenin—To determine whether EGF-R-mediated phosphorylation regulates the interaction of MUC1 with c-Src and β -catenin, we incubated MUC1/CD with EGF-R and ATP and

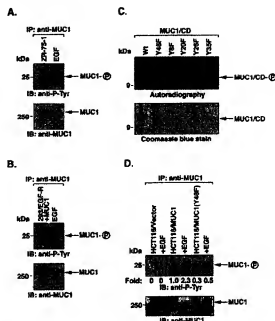


Fig. 3. EGF-R phosphorylates MUC1 *in vitro* and *in vivo*. A, lysates from ZR-75-1 cells treated with or without 10 ng/ml EGF for 5 min were subjected to immunoprecipitation with anti-MUC1. The immunoprecipitates were analyzed with anti-Tyr(P) (upper panel) and anti-MUC1 (lower panel). B, 293 cells were transiently transfected with MUC1 and MUC1. At 48 h, the cells were treated with or without 10 ng/ml EGF for 5 min. Lysates were subjected to immunoprecipitation with anti-MUC1. The immunoprecipitates were analyzed by immunoblotting with anti-Tyr(P) and anti-MUC1. C, purified wild-type MUC1/CD and the indicated mutant proteins were incubated with purified EGF-R and [γ -³²P]ATP. The reaction products were analyzed by SDS-PAGE and autoradiography (upper panel). Equal loading of the proteins was assessed by Coomassie Blue staining (lower panel). D, lysates from HCT116/vector, HCT116/MUC1, and HCT116/MUC1(Y46F) cells treated without or with 10 ng/ml EGF for 5 min at 37 °C were subjected to immunoprecipitation with anti-MUC1. The immunoprecipitates were analyzed by immunoblotting with anti-Tyr(P) and anti-MUC1. The signal intensities of the tyrosine-phosphorylated MUC1 proteins were compared with that of control HCT116/MUC1 (designated 1.0) by densitometric scanning. The relative intensities (mean \pm S.E.) from three separate experiments were: HCT116/vector, 0 \pm 0; HCT116/vector + EGF, 0 \pm 0; HCT116/MUC1, 1.0; HCT116/MUC1 + EGF, 2.2 \pm 0.3; HCT116/MUC1(Y46F), 0.5 \pm 0.3; and HCT116/MUC1(Y46F), 0.6 \pm 0.1.

then assessed binding to GST-Src-SH2 and GST- β -catenin. Immunoblot analysis of adsorbates to glutathione beads with anti-MUC1/CD showed that GST-Src SH2 binds to MUC1/CD following EGF-R phosphorylation (Fig. 4A). In addition, compared with MUC1/CD, there was substantially less binding of GST-Src-SH2 to the MUC1/CD(Y46F) mutant that had been incubated with EGF-R and ATP (Fig. 4A). Similar findings were obtained for binding of GST- β -catenin (Fig. 4A). To assess whether EGF-R-mediated phosphorylation of MUC1 induces binding of MUC1 to c-Src and β -catenin *in vivo*, anti-MUC1 immunoprecipitates from ZR-75-1 cells were analyzed by immunoblotting with anti-c-Src or anti- β -catenin. Analysis of lysates from control ZR-75-1 cells demonstrated a low but detectable interaction of MUC1 with c-Src and β -catenin (Fig. 4B). In concert with the *in vitro* results, stimulation of ZR-75-1 cells with EGF induced the interaction of MUC1 with c-Src and β -catenin (Fig. 4B). To confirm involvement of the MUC1 Tyr⁴⁶ site, HCT116 cells stably expressing wild-type MUC1 or MUC1(Y46F) were stimulated with EGF. Immunoblot analysis of anti-MUC1 immunoprecipitates with anti-c-Src demonstrated that, compared with wild-type MUC1, there was less EGF-induced binding

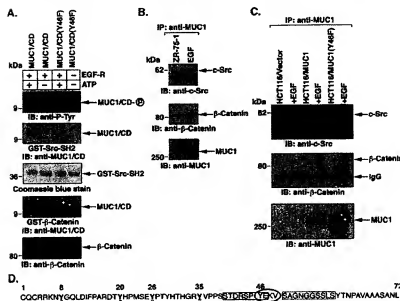


Fig. 4. EGF-R-mediated phosphorylation of MUC1 enhances the interaction of MUC1 with c-Src and β -catenin. **A**, purified MUC1/CD or MUC1/CD(Y46F) was incubated with EGF-R in the presence or absence of ATP for 30 min at 30 °C. GST-Src-SH2 or GST- β -catenin was then added, and the reaction was incubated for 1 h at 4 °C. Proteins precipitated with glutathione beads were separated by SDS-PAGE and subjected to immunoblot analysis with anti-MUC1/CD and anti-Tyr. Equal loading of the proteins was assessed by Coomassie blue staining or immunoblotting with anti- β -catenin. **B**, lysates from ZR-75-1 cells treated with or without 10 ng/ml EGF for 5 min were subjected to immunoprecipitation with anti-MUC1. The immunoprecipitates were analyzed by immunoblotting with anti-c-Src (upper panel), anti- β -catenin (middle panel), and anti-MUC1 (lower panel). **C**, HCT116/vector, HCT116/MUC1, and HCT116/MUC1(Y46F) cells were treated with or without 10 ng/ml EGF for 5 min at 37 °C. Lysates were subjected to immunoprecipitation with anti-MUC1. The immunoprecipitates were analyzed by immunoblotting with anti-c-Src (upper panel), anti- β -catenin (middle panel), and anti-MUC1 (lower panel). **D**, amino acid sequence of the MUC1 cytoplasmic tail. The GSK3 β binding and phosphorylation site (STDRSF), the c-Src SH2 binding motif (YEKV), and the β -catenin binding site (SAGNGGSSLS) are highlighted.

of MUC1(Y46F) to c-Src (Fig. 4C). Similar findings were obtained for β -catenin (Fig. 4C). These results show that EGF-R-mediated phosphorylation of MUC1 Y46 induces the interaction of MUC1 with c-Src and β -catenin.

MUC1 Integrates EGF-R, c-Src, and β -Catenin Signaling—The present findings and those recently reported for the mouse mammary gland (20) demonstrate that MUC1 interacts with EGF-R. The present results further show that EGF-R phosphorylates the MUC1 cytoplasmic tail on the YEKV motif. These findings and the recent demonstration that c-Src phosphorylates Tyr⁴⁶ (19) have supported regulation of the YEKV motif by both EGF-R and c-Src. The available evidence indicates that Tyr⁴⁶ is functionally important in regulating the interactions of MUC1 with multiple signaling pathways. EGF-R-mediated phosphorylation of MUC1 Tyr⁴⁶ functions as a binding site for the c-Src SH2 domain. Moreover, EGF stimulation is associated with increased binding of MUC1 and c-Src *in vivo*. EGF-R-mediated phosphorylation of MUC1 Tyr⁴⁶ also induces binding of MUC1 with β -catenin *in vitro* and in cells. In addition, phosphorylation of MUC1 Tyr⁴⁶ by EGF-R or c-Src down-regulates the interaction of MUC1 and GSK3 β (Ref. 19 and data not shown). In concert with these findings, the YEKV motif resides between the GSK3 β binding and phosphorylation site (STDRSF) (18) and the β -catenin binding site (SAGNGGSSLS) (14) (Fig. 4D). Taken together, the present results and those of previous studies (14, 18–20) support a model in which EGF stimulation induces phosphorylation of MUC1 on Tyr⁴⁶ and thereby integrates signaling among the c-Src, β -catenin, and GSK3 β pathways. The aberrant overexpression of MUC1 in human carcinoma cells could thus contribute to the transformed phenotype by dysregulation of EGF-R, c-Src, β -catenin, and/or GSK3 β signaling.

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Interaction of the DF3/MUC1 Breast Carcinoma-associated Antigen and β -Catenin in Cell Adhesion*

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The DF3/MUC1 mucin-like glycoprotein is aberrantly overexpressed in human breast carcinomas. The functional role of DF3 is unknown. The present studies demonstrate that DF3 associates with β -catenin. Similar findings have been obtained for γ -catenin but not α -catenin. DF3, like E-cadherin and the adenomatous polyposis coli gene product, contains an SXXXXSSL site that is responsible for direct binding to β -catenin. The results further demonstrate that interaction of DF3 and β -catenin is dependent on cell adhesion. These findings and the role of β -catenin in cell signaling support a role for DF3 in the adhesion of epithelial cells.

The human DF3 (*MUC1*, *episialin*, *PEM*) gene encodes a high molecular mass membrane-associated glycoprotein with a mucin-like external domain. The DF3 glycoprotein is expressed on the apical borders of secretory mammary epithelial cells and aberrantly expressed over the entire surface of carcinoma cells (1). The ectodomain consists of varying numbers of 20-amino acid tandem repeats that are subject to O-glycosylation and that contribute to the expression of a polymorphic protein (2-4). The N-terminal region contains hydrophobic signal sequences that vary as a consequence of alternate splicing (5-7). The C-terminal region includes a transmembrane domain and a 72-amino acid cytoplasmic tail that contains tyrosine phosphorylation sites (8, 9). The function of DF3 is unclear. However, high levels found on carcinoma cells reduce cell-cell and cell-extracellular matrix adhesion in a nonspecific manner (10-12). These studies have suggested that DF3 interferes with cellular adhesion by steric hindrance from the rigid ectodomain (11).

Cadherin cell adhesion molecules form complexes with the cytoplasmic α -, β -, and γ -catenin proteins (13). α -Catenin is required for cadherin-mediated cell adhesion and links cadherins to the actin cytoskeleton (14, 15). β -Catenin links α -catenin to the cadherins and is highly related to plakoglobin (γ -catenin) (16-18). β -Catenin is homologous to the *Drosophila* segment polarity gene product Armadillo (19) that acts downstream of Wnt/leucine (20). Armadillo forms complexes with *Drosophila* E-cadherin and α -catenin (21, 22). These findings have supported a role for β -catenin in morphogenetic signals. Other studies have demonstrated that β -catenin binds directly to the adenomatous polyposis coli (APC)³ gene product (23-25). The APC protein and E-cadherin form independent complexes with

β -catenin (25). γ -Catenin mediates similar interactions among APC, α -catenin, and the cytoskeleton (16).

The present results demonstrate that DF3 interacts directly with β -catenin. An SXXXXSSL motif in the DF3 cytoplasmic domain is responsible for binding to β -catenin. We also demonstrate that cell adhesion induces the interaction between DF3 and β -catenin.

MATERIALS AND METHODS

Cell Culture.—Human ZR-75-1 breast carcinoma cells were grown in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 100 μ g/ml streptomycin, 100 units/ml penicillin, and 2 mM L-glutamine. Cells were grown in suspension (0.3×10^6 to 10^7 cells/ml) with gentle rocking or as a monolayer on polystyrene culture dishes.

Cell Lysates.—Cells (~70% confluent) were lysed in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.6, 0.5% Brij 97, 10 μ M leupeptin, 10 μ M/ml aprotinin, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol) for 30 min on ice.

Lysates were cleared by centrifugation at 14,000 \times g for 15 min.
Immunoprecipitation and Immunoblotting.—Lysates were incubated with monoclonal antibody (mAb) DF3 (1), anti- β -catenin (Zymed Laboratories, Inc., San Francisco, CA), anti- β -catenin (Zymed), anti- α -catenin (Zymed), or anti-E-cadherin (Transduction Laboratories, Lexington, KY) for 2 h at 4 °C. Immunoprecipitates were prepared by incubation with rabbit anti-mouse IgG (Upstate Biotechnology, Inc., Lake Placid, NY) and protein A-Sepharose (Pharmacia Biotech Inc.) for 1 h at 4 °C. The precipitates were subjected to electrophoresis in 7.5% or 8% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes by dry transfer. The membranes were blocked in 5% nonfat dry milk in phosphate-buffered saline containing 0.05% Tween 20 and then incubated with an appropriate antibody for immunoblot analysis. Reactivity was detected by horseradish peroxidase-conjugated second antibodies and chemiluminescence (ECL, Amersham Corp.).

Direct Binding Studies.—The GST fusion construct expressing the DF3 cytoplasmic domain (CD) was prepared by polymerase chain reaction cloning and ligation into the pGEX2T vector. GST or GST-DF3/CD was affinity-purified with glutathione-Sepharose 4B beads and suspended in elution buffer (50 mM Tris-HCl, pH 8.0, 5 mM glutathione). Nitrocellulose filters were incubated with GST or GST-DF3/CD for 1.5 h at room temperature. Reactivity was detected with an anti-GST antibody (Santa Cruz Biotechnology).

RESULTS AND DISCUSSION

To identify proteins that associate with DF3, we analyzed mAb DF3 immunoprecipitates by SDS-PAGE and silver staining. The detection of a coprecipitated protein of 92 kDa was supported by reactivity with an antibody against β -catenin (Fig. 1A). Since E-cadherin forms complexes with α -, β -, and γ -catenins (26), we analyzed anti-DF3 immunoprecipitates for an association with α - and γ -catenins. While there was no detectable α -catenin in the precipitates, the results indicate that DF3 forms complexes with γ -catenin (Fig. 1, B and C). In the reciprocal experiments, anti-catenin immunoprecipitates were analyzed by immunoblotting with anti-DF3. The findings confirm binding of DF3 to β - and γ -catenins (Fig. 1D). As previously shown (26), E-cadherin formed complexes with all three of the catenins (Fig. 1D).

To determine if binding to DF3 is direct, we subjected anti- β -

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³ The abbreviations used are: APC, adenomatous polyposis coli; mAb, monoclonal antibody; CD, cytoplasmic domain; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis.

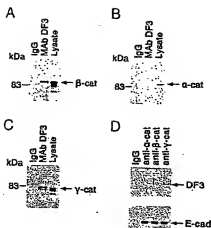


FIG. 1. Association of DF3 with β -catenin (β -cat) and γ -catenin. Lysates from adherent ZR-75-1 cells were subjected to immunoprecipitation with mAb DF3. The immunoprecipitates were analyzed for reactivity with anti- β -catenin (A), anti- γ -catenin (B), and anti- γ -catenin (C). Lysates were directly analyzed by immunoblotting as controls. D, lysates were subjected to immunoprecipitation with the indicated antibodies. The precipitates were analyzed by immunoblotting with mAb DF3 (upper panel) or anti-E-cadherin (E-cad, lower panel).

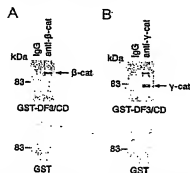


FIG. 2. Direct binding of DF3 to β -catenin (β -cat) and γ -catenin. Lysates were subjected to immunoprecipitation with anti- β -catenin (A) or anti- γ -catenin (B). The immunoprecipitates were separated by SDS-PAGE, and the proteins were transferred to nitrocellulose filters. The filters were incubated with GST or GST-DF3/CD and then washed and analyzed for reactivity with anti-GST.

catenin immunoprecipitates to SDS-PAGE and then transferred the separated proteins to filters. Incubation of the filters with a GST fusion protein that contains the DF3 cytoplasmic domain (GST-DF3/CD) demonstrated binding to β -catenin (Fig. 2A). By contrast, there was no detectable binding to GST (Fig. 2A). Similar results were obtained for γ -catenin (Fig. 2B).

Previous studies have demonstrated that β -catenin binds to SXXXXXSSL sites in E-cadherin (amino acids 840–848) and APC (seven motifs) (23, 24, 27) (Fig. 3A). β -Catenin also associates with the epidermal growth factor receptor, which contains a SITPLLSLS (amino acids 1030–1039) site (28). A similar site is present at amino acids 1239–1243 in DF3 (Fig. 3A). To assess whether β -catenin binds to the SXXXXXSSL site in DF3, we subjected cell lysates to immunoprecipitation with mAb DF3 in the presence of the synthetic peptide GGSSLSY. The results demonstrate that the peptide inhibits binding of β -catenin and DF3 (Fig. 3B). By contrast, there was no detectable effect on this interaction when using an irrelevant peptide (Fig. 3B). The GGSSLSY peptide also blocked interaction of DF3 and γ -catenin (Fig. 3B). These findings suggested that β - and γ -catenin bind to DF3 at similar sites.

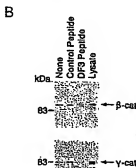
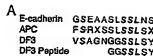


FIG. 3. DF3 binds to catenins at an SXXXXXSSL site. A, SXXXXXSSL sites in E-cadherin, APC, and DF3. B, lysates were subjected to immunoprecipitation with mAb DF3 in the presence of no added peptide, a control peptide (50 μ M; EAPPKIPEDKQ), or a 50 μ M GGSSLSY peptide. The immunoprecipitates were analyzed by immunoblotting with anti- β -catenin (β -cat) or anti- γ -catenin.

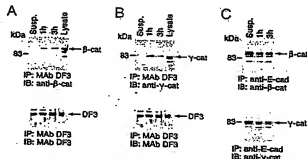


FIG. 4. Cell adhesion induces binding of DF3 with β -catenin (β -cat) and γ -catenin. Cells were trypsinized and grown in suspension by gentle agitation for 6 h. Suspension cells were allowed to adhere to culture dishes for 1 and 3 h. Adherent cells were subjected to immunoprecipitation with mAb DF3 (A and B) or anti-E-cadherin (anti-E-cad, C). The immunoprecipitates were analyzed for reactivity with anti- β -catenin, anti- γ -catenin, or mAb DF3. Lysates were directly analyzed by immunoblotting as controls.

The functional role of the association between DF3 and β -catenin was studied in cells grown in suspension and then grown as a monolayer. There was no detectable β -catenin in the mAb DF3 immunoprecipitates prepared from the suspension cells. By contrast, binding of DF3 to β -catenin was detectable at 1 and 3 h of adherence (Fig. 4A). Cell adhesion was also associated with formation of a complex with DF3 and γ -catenin (Fig. 4B), but not α -catenin (data not shown). A similar analysis of E-cadherin immunoprecipitates demonstrated little if any difference in binding to β - or γ -catenin in suspension as compared with adherent cells (Fig. 4C).

β -Catenin is involved in the formation of adherens junctions of epithelial cells. The cell adhesion E-cadherin protein and the APC tumor suppressor gene product compete for binding to the arm repeats of β -catenin (16) that are also found in Armadillo, γ -catenin, and certain other junctional proteins (29). The present studies demonstrate that DF3 also binds directly to β -catenin and that the SXXXXXSSL motif in DF3 is responsible for

this interaction. Similar results were obtained with the highly related γ -catenin. Whereas the cytoplasmic domain of DF3/MUC1 is phosphorylated on tyrosine (8, 9), it is not known if tyrosine sites influence binding of catenins to the serine-rich motif. The formation of a complex between DF3 and β -catenin (or γ -catenin) may differ from those found in other β -catenin complexes. The interaction of E-cadherin or APC complexes to the cytoskeleton is mediated by binding of β -catenin to α -catenin (16). By contrast, there was little if any α -catenin in the complex of DF3 and β -catenin. Moreover, while E-cadherin forms a stable complex with β -catenin in suspension and adherent cells, the interaction of DF3 with β -catenin is detectable following cell adhesion. Similar findings were obtained for the interaction of DF3 and γ -catenin. These findings support a role for DF3 in the adhesion of cells and provide support for a novel interaction of DF3 with catenins.

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United States Patent [19][11] **Patent Number:** 5,851,775**Barker et al.**[45] **Date of Patent:** Dec. 22, 1998[54] **β -CATENIN, TCF-4, AND APC INTERACT TO PREVENT CANCER**

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[21] **Appl. No.:** 821,355[22] **Filed:** Mar. 20, 1997[51] **Int. Cl.⁶** C12Q 1/68; G01N 33/53[52] **U.S. Cl.** 435/6; 435/7.1; 435/189; 435/366[58] **Field of Search** 435/6, 36, 7.1, 435/189, 366[56] **References Cited****PUBLICATIONS**

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Primary Examiner—John L. LeGuyader
Assistant Examiner—Robert Schwartzman
Attorney, Agent, or Firm—Banner & Witcoff, Ltd.

[57] **ABSTRACT**

The APC tumor suppressor protein binds to β -catenin, a protein recently shown to interact with Tcf/Lef transcription factors. Here, the gene encoding a Tcf family member that is expressed in colonic epithelium (hTcf-4) was cloned and characterized. hTcf-4 transactivates transcription only when associated with β -catenin. Nuclei of APC^{-/-} colon carcinoma cells were found to contain a stable β -catenin-hTcf-4 complex that was constitutively active, as measured by transcription of a Tcf reporter gene. Reintroduction of APC removed β -catenin from hTcf4 and abrogated the transcriptional transactivation. Constitutive transcription of TCF target genes, caused by loss of APC function, may be a crucial event in the early transformation of colonic epithelium. It is also shown here that the products of mutant APC genes found in colorectal tumors are defective in regulating β -catenin/Tcf-4 transcriptional activation. Furthermore, colorectal tumors with intact APC genes were shown to contain subtle activating mutations of β -catenin that altered functionally significant phosphorylation sites. These results indicate that regulation of β -catenin is critical to APC's tumor suppressive effect and that this regulation can be circumvented by mutations in either APC or β -catenin.

9 Claims, 13 Drawing Sheets

FIG. 1A

hTCF-4E

hTCF-1E

[illegible]

FIG. 1B

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194 P P H L P A D V D P K T G I P R P P H P P D I S P Y Y P L S P G T V G Q I P H P
    | | | | | : | | | | | : | | | | | : | | | | |
173 T P A P A D I S Q K Q V H R P L Q T P D L S G F Y S L T S G S M G Q L P H T
    | | | | | : | | | | | : | | | | | : | | | | |
234 L G W L V P Q Q G Q V Y P I T T G G F R H P Y P T A L T V N A S V S R F
    : | | | | | : | | | | | : | | | | | : | | | | |
211 V S W P S P P L Y P L S P S C G Y R Q H F F A P T A A P G A P Y P R F T H
    | | | | | : | | | | | : | | | | | : | | | | |
271 P P H M V P P H H T L H T T G I P H P A I V T P T V K Q E S S Q S D V G S L H S
    | | | | | : | | | | | : | | | | | : | | | | |
248 P S L M L G S G V P G H P A A I P H P A I V P P S G K Q E L Q P F D R N L
    | | | | | : | | | | | : | | | | | : | | | | |
311 S K H Q D S K K E E K K K P H I K K P L N A F M L Y M K E M R A K V V A E C
    | | | | | : | | | | | : | | | | | : | | | | |
285 K T Q A E S K A E K E A K K P T I K K P L N A F M L Y M K E M R A K V I A E C
    | | | | | : | | | | | : | | | | | : | | | | |
350 T L K E S A A I N Q I L G R R W H A L S R E E Q A K Y Y E L A R K E R Q L H M Q
    | | | | | : | | | | | : | | | | | : | | | | |
324 T L K E S A A I N Q I L G R R W H A L S R E E Q A K Y Y E L A R K E R Q L H M Q
    | | | | | : | | | | | : | | | | | : | | | | |
390 L Y P G W S A R D N Y G K K K K R K R D K Q P G E T N E H S E C F L N P C L S L
    | | | | | : | | | | | : | | | | | : | | | | |
364 L Y P G W S A R D N Y G K K K R R S R E K H Q E S
    | | | | | : | | | | | : | | | | | : | | | | |
430 P P I T D L S A P K K C R A R F G L D Q Q N N W C G P C R R K K K C V R Y I Q G
    | | | | | : | | | | | : | | | | | : | | | | |
389 T T D P G S P K K C R A R F G L N Q Q T D W C G P C R R K K K C I R Y L P G
    | | | | | : | | | | | : | | | | | : | | | | |

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FIG. 1C

470 EGSCLSPPSSDGLSDSPSPSPNLLGSPPPRDAKSKQTEQT
| | | | | | | | | | | | | | | | | | | |
427 EGRCPSPVPSSDLSAL GCGSPAPQDS PSYHLLPRFPTE
| | | | | | | | | | | | | | | | | | | |
510 PLSLSLKPDPLAHLSSMMPPPPALLAEATHKASALCP
: | | | | | | | | | | | | | | | | | |
465 LLSPAEAPATSPGLSTALSLETPGPPQAPRSTLQSTVQ
| | | | | | | | | | | | | | | | | | | |
547 NGALDLPALQPAAPSSSIAQPSSTSWLHSHSSLAGTQPP
: | | | | | | | | | | | | | | | | | |
505 QQESQRQVA*
| | | | | | | | | | | | | | | | | | | |
587 PLSLVTKSLE*

hTCF-4B

hTCF-1B

3390	LYPGWSARDNY	GKKKKRKR	DDKQPG	ETNGEX	KSAPAT	YKVK
3384	LYPGWSARDNY	GKKKKR	SR	KKHQ	ESTT	GKRNAPGTYP
430	AAASAHPLQMEAY	*				
404	AAAPAPFLPMTVL	*				

FIG. 2A

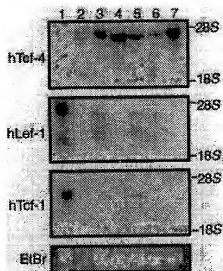


FIG. 2B

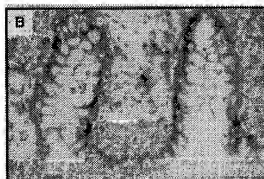


FIG. 2C



FIG. 3A

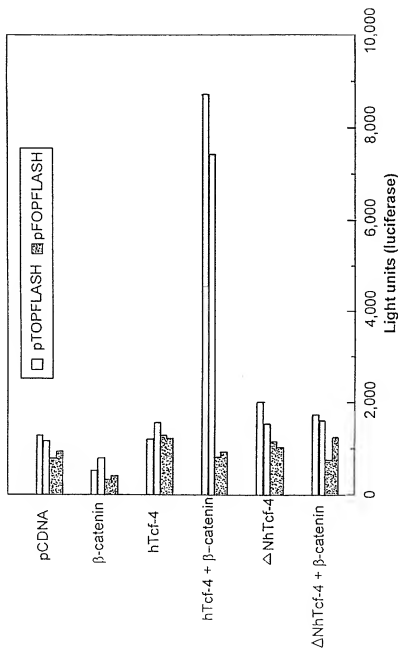


FIG. 3B

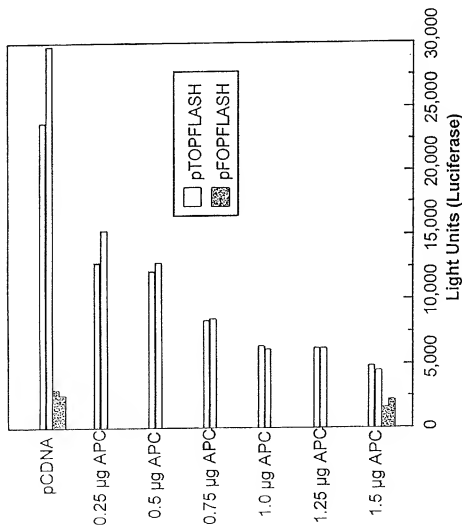


FIG. 3C

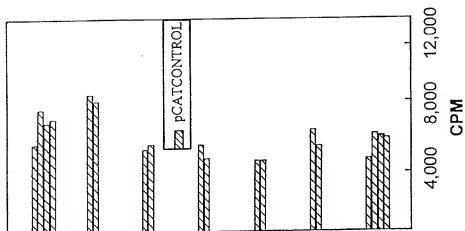


FIG. 4

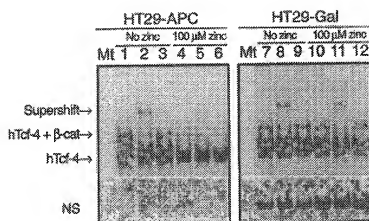


FIG. 5A

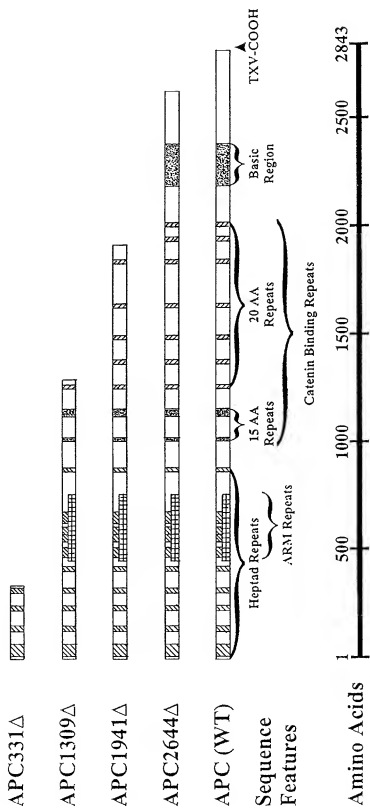


FIG. 5B

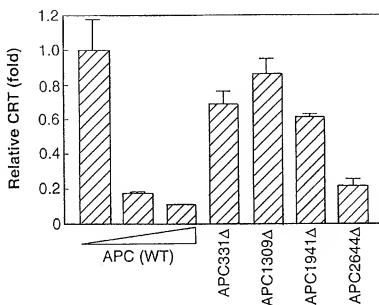


FIG. 6B

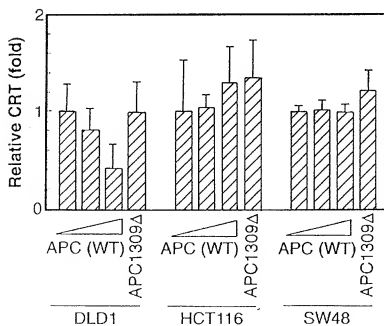


FIG. 6A

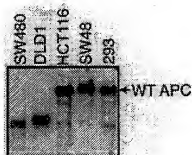


FIG. 7A

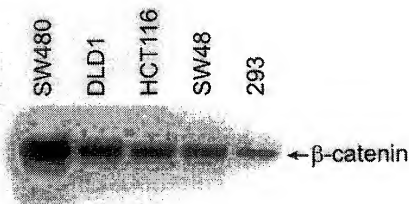


FIG. 8A

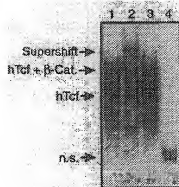


FIG. 7B

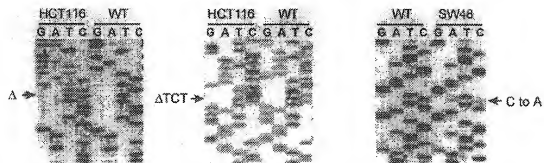


FIG. 7C

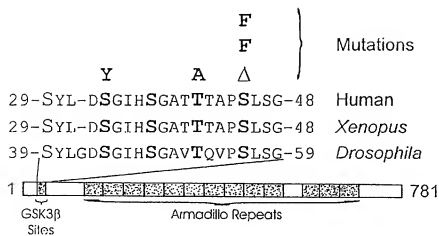
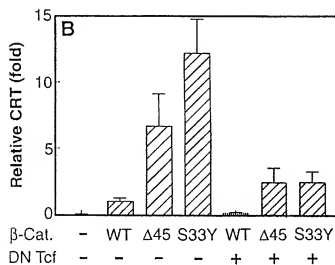


FIG. 8B



β-CATENIN, TCF-4, AND APC INTERACT TO PREVENT CANCER

The U.S. Government has a paid-up license in this invention and the right in limited circumstances to require the patent owner to license others on reasonable terms as provided for by the terms of grant CA57345 awarded by the National Institutes of Health.

TECHNICAL FIELD OF THE INVENTION

This invention is related to the field of cancer diagnostics and therapeutics. More particularly it relates to methods for diagnosing and treating cancers associated with APC or β-catenin mutations.

BACKGROUND OF THE INVENTION

Mutations of the adenomatous polyposis coli (APC) gene are the most common disease-causing genetic events in humans; approximately 50% of the population will develop colorectal polyps initiated by such mutations during a normal life span (14). Individuals who inherit APC mutations develop thousands of colorectal tumors, consistent with APC's tumor suppressor or "gatekeeping" role in colorectal tumorigenesis (15,16). APC homodimerizes through its amino-terminus (17), and interacts with at least six other proteins: β-catenin (18), γ-catenin (plakoglobin) (19), tubulin (20), EBI (21), hDLG, a homologue of a Drosophila tumor suppressor protein (22), and ZW3/GSK3β kinase (23). Whether any of these interacting proteins communicate APC growth-controlling signals is unknown. Thus there is a need in the art for a fuller understanding of how the tumor suppressor gene APC functions in cells.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide human nucleotide sequences encoding transcriptional activation proteins.

It is another object of the present invention to provide isolated preparations of transcriptional activation proteins.

It is an object of the present invention to provide methods of determining the presence or absence in a cell of wild-type APC or a downstream protein in the APC transcription regulatory pathway.

Another object of the invention is to provide methods of identifying candidate drugs for use in Familial Adenomatous Polyposis (FAP) patients or patients with increased risk of developing cancer.

It is yet another object of the invention to provide methods of identifying candidate drugs for the treatment of cancer patients, in particular those with APC or β-catenin mutations.

Another object of the invention is to provide a method for diagnosing cancer in a sample suspected of being neoplastic.

Another object of the invention is to provide a method for treating a patient with colorectal cancer or other cancer associated with FAP.

These and other objects of the invention are achieved by providing one or more of the embodiments described below. In one embodiment of the invention an intron-free DNA molecule is provided which encodes Tcf-4 protein as shown in SEQ ID NO: 5 or 6.

According to another embodiment of the invention an isolated Tcf-4 protein is provided. The protein is substantially free of other human proteins, and has a sequence as shown in SEQ ID NO: 5 or 6.

In another embodiment of the invention a method is provided for determining the presence or absence in a cell of wild-type APC or a downstream protein in the APC transcription regulatory pathway. The method comprises the steps of:

introducing a Tef-responsive reporter gene into the cell; and measuring transcription of said reporter gene; wherein a cell which supports active transcription of said reporter gene does not have wild-type APC or does not have a wild-type downstream protein in the APC transcription regulatory pathway.

According to yet another embodiment of the invention a method is provided for determining the presence or absence in a cell of wild-type APC. The method comprises the steps of:

contacting a Tcf-responsive reporter gene with a lysate of the cell; and measuring transcription of said reporter gene; wherein a lysate which inhibits said transcription has wild-type APC.

In still another embodiment of the invention a method of identifying candidate drugs is provided. The drugs may be useful for treatment of FAP or other cancer patients or patients with increased risk of developing cancer. The method comprises the steps of:

contacting a cell having no wild-type APC or a mutant p-catenin with a test compound; measuring transcription of a Tcf-responsive reporter gene, wherein a test compound which inhibits the transcription of the reporter gene is a candidate drug for cancer therapy.

According to yet another aspect of the invention another method is provided for identifying candidate drugs for use in for use in FAP patients, colon cancer patients, patients with mutations in β-catenin or APC, or patients with increased risk of developing cancer. The method, comprises the steps of:

contacting a Tcf-responsive reporter gene with a test compound under conditions in which the reporter gene is transcribed in the absence of the test compound; and measuring transcription of the Tcf-responsive reporter gene; wherein a test compound which inhibits said transcription is a candidate drug for cancer therapy.

According to another aspect of the invention a method is provided for identifying candidate drugs for use in FAP patients or patients with increased risk of developing cancer. The method comprises the steps of:

contacting a test compound with β-catenin and Tcf-4 under conditions in which β-catenin and Tcf-4 bind to each other; and

determining whether the test compound inhibits the binding of β-catenin and Tcf-4, a test compound which inhibits the binding being a candidate for cancer therapy or prophylaxis.

According to still another embodiment of the invention a method is provided for diagnosing cancer in a sample suspected of being neoplastic, the method comprises the steps of:

comparing a CTNNB sequence found in the sample to a second CTNNB sequence found in a normal tissue, wherein a difference between the first and second sequence is an indicator of cancer.

According to another aspect of the invention a method is provided for treating a patient with colorectal cancer or other cancer associated with FAP.

The method comprises the step of:

administering to the patient a nucleotide sequence comprising a portion of the APC coding sequence, said portion consisting of the β -catenin binding site.

According to another aspect of the invention a method is provided for treating a patient with colorectal cancer or other cancer associated with FAP. The method comprises the step of:

administering to the patient a polypeptide comprising a portion of the APC coding sequence, said portion consisting of the β -catenin binding site.

The present invention thus provides the art with diagnostic, therapeutic and drug discovery methods especially useful for FAP and other cancers with APC or β -catenin mutations.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Sequence comparison of hTcf-4 and hTcf-1.

Two alternative splice forms of hTcf-4 were identified, each encoding a different COOH-terminus. One form (hTcf-4E; SEQ ID NO:6) was homologous to hTcf-1E; SEQ ID NO:9 (FIG. 1A) (7); the other form (hTcf-4B; SEQ ID NO:5) was homologous to hTcf-1B; SEQ ID NO:8. (FIG. 1B). The highly conserved NH₂-terminal interaction domain and the High Mobility Group (HMG) box DNA-binding region are boxed. Abbreviations for the amino acids are: A: Ala; C: Cys; D: Asp; E: Glu; F: Phe; G: Gly; H: His; I: Ile; IC: Lys; L: Leu; M: Met; N: Asn; P: Pro; Q: Gln; R: Arg; S: Ser; T: Thr; V: Val; W: Trp; and Y: Tyr.

FIG. 2. Analysis of hTcf-4 expression in colonic epithelium.

(FIGS. 2A, 2B, and 2C) Northern blot analysis of hTcf-4, hTcf-1, hTcf-1 expression in Jurkat T cells (lane 1); colonic mucosa (lane 2); colon carcinoma cell lines DLD-1 (lane 3), HCT116 (lane 4); SW480 (lane 5); SW620 (lane 6); HT29 (lane 7). Lane 2 contains 5 μ g total RNA; all others contain 15 μ g total RNA. The positions of 18S and 28S ribosomal RNAs are shown. EtBr, ethidium bromide stain. (FIG. 2B) In situ hybridization of healthy human colon tissue to an hTcf-4 probe. (FIG. 2C) In situ hybridization to a negative control probe (a fragment of the *E. coli* neomycin resistance gene).

FIGS. 3A, 3B. Transactivational properties of β -catenin/hTcf-4.

All reporter assays were performed as duplicate transfections. For each condition, both values are shown. (FIG. 3A) Reporter gene assays in IIA-1.6 B cells. Cells were transfected by electroporation with 1 μ g luciferase reporter plasmid, 5 μ g β -catenin expression plasmid, and 3 IIA-1.6 expression plasmids. Empty pCDNA was added to a total of 10 μ g plasmid DNA. (FIG. 3B) Reporter gene assays in SW480 colon carcinoma cells. Cells were transfected with 0.3 μ g of the indicated luciferase reporter gene, 0.7 μ g pCATCONTROL as internal control, the indicated amounts of pCMVNeoAPC, and empty pCDNA to a total of 2.5 μ g plasmid DNA. Control CAT values are given in the right panel.

FIG. 4. Constitutive presence of β -catenin-hTcf-4 complexes in APC⁺ cells. Gel retardation assays were performed on nuclear extracts from the indicated cell lines before and after a 20-hour exposure to Zn²⁺. Samples in lanes 1, 4, 7, 10 were incubated under standard conditions. To the samples in lanes 2, 5, 8, 11, 0.25 μ g anti- β -catenin was added. To the samples in lanes 3, 6, 9, 12, 0.25 μ g of a control (human CD4) antibody was added. N.S., nonspecific band also observed with mutant (nonbinding) probe (lane M).

FIGS. 5A and 5B. Effects of APC mutations on CRT. (FIG. 5A) Schematics of wild-type (WT) and mutant APC. APC is a 2843-amino-acid (AA) protein (32) with conserved armadillo (ARM) repeats in the amino-terminus (33), 15 and

20 AA β -catenin-binding repeats in the central region (18, 19), and a basic region in the carboxyl-terminus (32). The carboxyl-terminus also contains a TVV sequence which mediates DLG binding (22). (FIG. 5B) Effects of WT and mutant APC on CRT. SW480 cells containing endogenous mutant APC were transfected with the APC expression vectors shown in (FIG. 5A) and CRT was measured. Cells were transfected with increasing amounts of WT APC (0, 0.15 and 0.5 μ g) or 0.5 μ g mutant APC. CRT reporter activities are expressed relative to assays containing no WT APC and are the means of three replicates. Error bars represent standard deviations.

Lipofectamine was used to cotransfect SW480 cells with an internal control (0.5 μ g pCMV- β gal), a reporter construct (0.5 μ g pTOPFLASH or pOPFLASH) and the indicated amount of the various APC expression vectors. The pTOPFLASH reporter contained an optimized Tcf-binding site 5' of a luciferase reporter gene, whereas pOPFLASH contained a mutated site that does not bind Tcf. The amount of DNA in each transfection was kept constant by addition of an appropriate amount of empty expression vector (pCPEA).

Luciferase and β -galactosidase activities were determined 16 hours after transfection. Luciferase activity was corrected for transfection efficiency (using the control β -galactosidase activity) and nonspecific transcription (using the pOPFLASH control).

FIGS. 6A and 6B. Evaluation of CRT in colorectal cancer cell lines with WT APC. (FIG. 6A) Immunoblot of endogenous APC in the DLD1, SW480, HCT116, SW48 and 293 cell lines, developed with APC monoclonal antibody F69 (34). (FIG. 6B) Effects of exogenous WT APC on CRT in cell lines with endogenous mutated or WT APC. Cells were transfected with increasing amounts (0, 0.15 μ g, 0.5 μ g for DLD1 and SW48; 0, 0.5 μ g, 5 μ g for HCT116) of WT APC or APC1309A mutant (0.5 μ g for DLD1 and SW48; 5 μ g for HCT116) and CRT was assessed as in FIG. 5. CRT reporter activities are expressed relative to activity in extracts without exogenous APC and are the means of three replicates. Error bars represent standard deviations.

FIGS. 7A, 7B and 7C. Evaluation of β -catenin in colorectal cancer cell lines with WT APC. (FIG. 7A) Immunoblot of the cell lines used in this study, developed with β -catenin monoclonal C19220 (Transduction Laboratories, Lexington, Ky) (31). (FIG. 7B) Sequence of CTNNB1 in HCT116 and SW48. Overlapping sequences constituting the entire CTNNB1 were amplified by RT-PCR from SW480, DLD1, HCT116, and SW48 cells, and sequenced directly with ThermoSequenase (Amersham). In the case of HCT116, a PCR product containing the deleted region was also cloned into pCI-neo (Promega, Madison) and multiple clones corresponding to each allele were individually sequenced.

The left panel (nts 121 to 143 from HCT116) reveals the presence of a deletion in addition to the WT sequence. The middle panel (antisense strand 156 to 113 of the WT and deleted alleles of HCT116) reveals the 3-bp deletion (Δ TC) that removed codon 45 in half the clones. The right panel (nts 80 to 113 from SW48) reveals a C to A transition affecting codon 33 (TCT to TAT). FIG. 7C) Schematic of β -catenin illustrating the armadillo repeats (33) in human (SEQ ID NO:10), Xenopus (SEQ ID NO:10) and drosophila (SEQ ID NO:11) and negative regulatory domain. The residues in larger type fit the consensus sequence for GSK3 β

phosphorylation (29) and those in bold have been demonstrated to affect down regulation of β -catenin through GSK3 β phosphorylation in *Xenopus* embryos (27). The five mutations found in human colon cancers are indicated at the top.

FIGS. 8A and B. Functional evaluation of β -catenin mutants. (FIG. 8A) Constitutive nuclear complex of β -catenin and Tcf in HCT116 cells. The presence of nuclear β -catenin-Tcf complexes was assessed by gel shift assays. Lanes 1 to 3, optimal Tcf retardation probe shifted with nuclear extract from HCT116 cells with addition of no antibody (lane 1), anti β -catenin (0.25 μ g, lane 2), or an irrelevant antibody (0.25 μ g, lane 3). Lane 4, mutant Tcf retardation probe shifted with nuclear extract from HCT116 cells. n.s., nonspecific shifting seen with the mutant probe. (FIG. 8B) Effects of the β -catenin mutations on CRT: 293 cells were transfected with WT (WT) or mutant (Δ 45, S33Y) β -catenin and CRT was assessed. CRT reporter activities are expressed relative to WT β -catenin and are the means of three replicates. Error bars represent standard deviations. β -catenin expression constructs were prepared as follows. WT CTNNB1 was amplified by RT-PCR from SW480 cells and cloned into the mammalian expression vector pCI-neo (Promega) to produce pCI-neo- β -cat. The pCI-neo- β -cat Δ 45 and S33Y were generated by replacing codons 1 to 89 in pCI-neo- β -cat with a PCR product encoding the equivalent region from HCT116 or SW48 cDNA, respectively. The structures of all constructs were verified by sequence analysis. Lipofectamine was used to cotransfect 293 cells with an internal control (0.1 μ g CMV-Flag), a reporter (0.5 μ g pTOPFLASH or pOPFLASH), a Tcf-4 expression vector (0.5 μ g pCDNA-Tcf-4), and β -catenin (0.5 μ g) or dominant negative hTcf-4 (1.0 μ g) expression vectors. CRT was determined as described above.

DETAILED DESCRIPTION

It is a discovery of the present invention that hTcf-4 binds to β -catenin and activates transcription in colorectal epithelial cells. Moreover, it has now been found that APC regulates this transcriptional activation, at least in part by binding to β -catenin. In colorectal cancer cells this regulation is frequently abrogated, either by mutation of APC or by mutation of β -catenin.

Two alternative splice forms of human Tcf-4 have been found. One form (hTcf-4E) is homologous to hTcf-1E and the other (hTcf-4B) is homologous to hTcf-1B. The sequence of the nucleotide and amino acid sequences are shown in SEQ ID NOS: 1, 2, 5, and 6. The coding sequences and proteins can be used in assays as described below. Intron-free DNA molecules are provided which are originally made by reverse transcription of a mRNA molecule. They can be propagated in cells or amplified as is desired. Isolated Tcf-4 proteins can be provided substantially free of other human proteins if, for example, the nucleotide sequences are expressed in non-human cells. Methods and vectors for achieving such expression are well known in the art. Choice of such expression means is made by the skilled artisan according to the desired usage and convenience.

Cells can be tested to determine if they have a wild-type APC or a wild-type downstream protein in the APC transcription regulatory pathway, called herein the CRT pathway (β -catenin/Tcf-regulated transcription). One protein within the CRT pathway which has been identified as a target of mutations in human cancers is β -catenin (encoded by the CTNNB1 gene). Other parts of the pathway are also likely

to be targets. Although the target genes of the CRT pathway have not been identified, they can be readily identified using the system disclosed here. Genes which are differentially transcribed in the presence of wild-type and mutant CTNNB1, for example, can be identified.

Tcf-responsive reporter genes are those constructs which comprise a readily detectable or assayable gene (such as luciferase, β -galactosidase, chloramphenicol acetyltransferase) linked in cis to a Tcf-responsive element. Such responsive elements are known in the art (7) and any such elements can be used. An optimal Tcf motif contains the sequence CCTTTGATC. From one to twenty copies, and preferably from three to six copies, of the motif may be used. Mutation of the sequence to CCTTTGGCC abrogates responsiveness. Another necessary part of such constructs is a minimal promoter, such as the c-Fos or the Herpes virus thymidine kinase promoter. Transcription of the reporter gene may be performed by any means known in the art, usually by assaying for the activity of the encoded gene, although immunological detection methods can also be used. In addition, transcription can be monitored by measuring the transcribed mRNA directly, typically using oligonucleotide probes.

As shown below, a cell which has a wild-type APC protein will inhibit CRT. However, most mutations in APC render APC unable to inhibit CRT. Similarly, certain mutations in CTNNB1 render β -catenin super-active and/or refractory to the inhibition by APC. Thus measuring Tcf-responsive reporter gene transcription is an indication of the status of APC and CTNNB1. Mutations in both of these genes are associated with cancers and therefore provides diagnostic and prognostic information.

Assays for CRT can be accomplished in vitro or in cells. If the assay is to be accomplished in cells, then a Tcf-responsive reporter gene must be introduced into the cell. Any means for introducing genetic material into cells can be used, including but not limited to infection, transfection, electroporation. If the assay is to be performed in vitro then the components for transcription must be present. These include suitable buffers, RNA polymerase, as well as ribonucleotides. If the protein product is to be assayed, then the components for translation must also be present, such as ribosomes, and amino acids.

These assays can also be used to screen compounds for potential as anti-cancer therapeutic agents. Using either the in vitro or cell form of the assay, test compounds can be introduced to determine whether they are able to mimic the effect of wild-type APC or to convert a mutant APC into a form which is able to inhibit CRT or a mutant β -catenin into a form which is regulatable by APC. In addition, compounds can be tested for the ability to inhibit the binding of β -catenin and Tcf-4, thus mimicking the action of APC. Such a test can be conducted in vitro or in vivo, for example using a two hybrid assay.

A means for diagnosis of cancers is the result of the observation that CTNNB1 mutations are found in tumor cells, especially those which have wild-type APC. Such mutations can be found, inter alia, by sequencing either the gene or the protein found in a sample. Functional assays can also be used, such as whether β -catenin binds to APC or Tcf-4, or whether it is capable of mediating CRT. Sequences can be compared to those found in a normal tissue of a human, especially the same human who provided the sample being tested. Suitable tumors for testing include, but are not limited to those which are associated with FAP. Suitable tumors include colorectal cancer, thyroid cancer, brain

cancer, medulloblastoma, desmoid tumor, osteoma, breast cancer, and head and neck cancer. Because APC mutations are so frequent, and because it appears that APC mutations do not occur in the same tumors as CTNNB1 mutations, one can prescreen samples for APC mutations before performing a CTNNB1 determination.

The portion of the APC gene which encodes the β -catenin binding site can be used in a gene therapy format. Suitable techniques are known in the art for administering genes to tumors, and any such technique can be used. Suitable expression vectors are also known in the art and it is within the skill of the artisan to select an appropriate one. Upon expression in a tumor cell of the β -catenin binding portion of APC, β -catenin will be bound and titrated away from binding to Tcf-4, thus preventing unregulated expression of the CRT target genes. Similarly, a polypeptide portion of APC containing the β -catenin binding site can be administered to cells to perform a titration of β -catenin. Techniques for such administration to cells is well known in the art. Cells which are treated with either the polynucleotide or the polypeptide can be used to study the interaction between APC and β -catenin, and for developing drugs which interfere with such binding.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only, and are not intended to limit the scope of the invention.

EXAMPLE 1

This example identifies Tcf-4 as the expressed family member in colorectal epithelial cells and provides the complete sequence of the cloned cDNA.

There are four known members of the Tcf/Lef family in mammals: the lymphoid-specific factors Tcf-1 and Lef-1 (7,8), and the less well characterized Tcf-3 and 4(9). We performed a qualitative Reverse Transcriptase-Polymerase Chain Reaction assay for expression of the four Tcf/Lef genes on 43 colon tumor cell lines. While most colon cell lines expressed more than one of the genes, only hTcf-4 mRNA was expressed in essentially all lines.

We then screened a human fetal CDNA library and retrieved clones encoding full-length hTcf-4 (FIG. 1). A genomic fragment encoding, the HMG box region of hTcf-4 (7) was used to probe a human 12 week-fetal cDNA library in Lambda GT-11. Positive clones were subcloned into pBluescriptSK and sequenced. See SEQ ID NOs: 1 and 2. The predicted sequence of hTcf-4 was most similar to that of hTcf-1. Alternative splicing yielded two COOH-termini that were conserved between hTcf-1 and hTcf-4. The NH₂-terminus, which in hTcf-1, mLeF-1 and Xenopus TCF-3 mediates binding to β -catenin (6), was also conserved in hTcf-4. Northern blot analysis of selected colon carcinoma cell lines revealed high-level expression of hTcf-4 (FIG. 2A). Northern blot hybridizations (7) were performed with full-length hTcf-1, hTcf-1, hTcf-4 and hTcf-4 cDNA. Colon epithelial cells were freshly prepared from a mucosal preparation dissected from a healthy surgical colon sample. The sample was minced, and incubated with 1 mM dithiothreitol (DTT) in Hanks' medium to remove mucus. Single-cell suspensions were prepared by incubation at RT in 0.75 mM EDTA in Hanks' medium. Epithelial cells were separated from lymphocytes by Percoll gradient centrifugation.

As evidenced by *in situ* hybridization (FIG. 2, B and C) and Northern blotting (FIG. 2A), hTcf-4 mRNA was readily detectable in normal colonic epithelium, whereas hTcf-1 and

hLef-1 were not detectable. *In situ* hybridization of 6 μ frozen sections of healthy colon biopsy samples were performed as described(10). hTcf-4 cDNA encoding amino acids 200 to 310 was amplified and labeled with Dig-11-dUTP (Boehringer Mannheim, Germany) by PCR. After hybridization and washing, the sections were sequentially incubated with mouse anti-Dig antibody (Boehringer) and a horseradish peroxidase conjugated rabbit antibody to mouse immunoglobulin (Dako, Glostrup, Denmark). The signal was visualized with diaminobenzidine, which produces a reddish-brown precipitate. Blue counterstaining was performed with haematoxyline.

EXAMPLE 2

This example demonstrates the interaction of Tcf-4 and β -catenin and their function as a transcriptional activating factor.

To investigate whether hTcf-4 functionally interacts with β -catenin, we used two sets of reporter constructs in a β -catenin-Tcf reporter gene assay (7). One contained three copies of the optimal Tcf motif CCTTTGATC, or three copies of the mutant motif CCTTTGGCC, upstream of a minimal c-Fos promoter driven-luciferase expression (PTOPFLASH and PPOFLASH). The second set contained three copies of the optimal motif, or three copies of the mutant motif, upstream of a minimal Herpes virus thymidine kinase promoter driven-Chloramphenicol Acetyl Transferase (CAT) expression (PTOPCAT and PPOPCAT, respectively). Reporter gene assays were performed as in (7). In brief, 2 \times 10⁵ cells were transfected with plasmids by electroporation. After 24 hours, cells were harvested and lysed in 1 mM DTT, 1 % Triton X-100, 15% glycerol, 25 mM Tris pH 7.8 and 8 mM MgCl₂. cDNAs encoding Myc-tagged versions of β -catenin and hTcf-4 were inserted into the mammalian expression vector pCDNA (Invitrogen). PCATCONTROL, encoding the CAT enzyme under the control of the SV40 promoter, was purchased from Promega.

Epitope-tagged hTcf-4 and a deletion mutant lacking, the NH₂-terminal 30 amino acids (Δ NhTcf-4) were cloned into the expression vector pCDNA. Transient transfections were performed in a murine B cell line (HAI.6), that does not express any of the Tcf genes (6).

The TOPFLASH reporter was strongly transcribed upon cotransfection with the combination of β -catenin and hTcf-4 plasmids, but not with the individual plasmids or with the combination of β -catenin and Δ NhTcf-4 plasmids. No enhanced transcription was detected in cells transfected with the negative control PPOFLASH (FIG. 3A). These results show that interaction of the NH₂-terminus of hTcf-4 with β -catenin results in transcriptional activation.

EXAMPLE 3

This example demonstrates the functional regulation of CRT transcriptional activation by wild-type APC.

In three APC⁺ carcinoma cell lines, SW480, SW620 and DLD-1 (FIG. 3B), the PTOFLASH reporter was 5-20 fold more actively transcribed than PPOFLASH. Importantly, transfection of SW480 cells with the reporter gene and an APC-expression vector abrogated the transcriptional activity in a dose-dependent manner (FIG. 3B). In contrast APC had no effect on a cotransfected internal control (pCATCONTROL), or on the basal transcription of PPOFLASH (FIG. 3B). The use of PTOFLASH and PPOPCAT instead of PTOFLASH and PPOFLASH led to comparable observations. The constitutive transcriptional activity of Tcf reporter genes in APC⁺ colon carcinoma cells was in

stark contrast to the inactivity of these genes in non-colonic cell lines, including IIAL6 B cells (FIG. 3A), the C57MG breast carcinoma cell line, the Jurkat and BW5147 T cell lines, the Daudi and NS1 B cell lines, the K562 erythromyocloid cell line, the HcLa cervical carcinoma line, the HepG2 hepatoma cell line; 3T3, 3T6, and Rat-1 fibroblasts; and the kidney derived SV40-transformed COS cell line (7,16).

EXAMPLE 4

This example demonstrates that a functional β -catenin-hTcf-4 complex exists constitutively in APC^{-/-} cells.

We used HT29-APC^{-/-} colon carcinoma cells (12), in which APC is controlled by a metallothionein promoter. Induction by Zn²⁺ restores wild-type levels of APC, and leads to apoptosis (12). HT29-Gal cells which carry a Zn²⁺-inducible LacZ gene were used as a control. The only Tcf family member expressed in HT29 is hTcf-4 (FIG. 2C). In nuclear extracts from uninduced HT29 derived transfectants, we readily detected hTcf-4 by gel retardation (FIG. 4). An additional band of slightly slower mobility was also observed. The addition of a β -catenin antibody resulted in the specific retardation of the latter band, indicating that it represented a β -catenin-hTcf-4 complex (FIG. 4) (12). After Zn²⁺ induction for 20 hours, the β -catenin-hTcf-4 complex was diminished sixfold relative to uncomplexed hTcf-4 in HT29-APC1, while no significant change was observed in HT29-Gal cells (FIG. 4). Importantly, the overall levels of cellular β -catenin do not change during the induction period in HT29-APC1 cells (12).

Gel retardation assays were performed as described elsewhere (7). Extracts were prepared from intact nuclei that were washed four times to avoid contamination with cytoplasmic β -catenin. As the optimal Tcf/Lef probe, we used a double-stranded 15-mer CCCTTTGATCTTACC (SEQ ID NO:3); the control probe was CCCTTTGGCCTTACC (SEQ ID NO:4). (All oligonucleotides were from Isogene, Holland). The β -catenin antibody was purchased from Transduction Laboratories Lexington, Ky.). A typical binding reaction contained 3 μ g nuclear protein, 0.1 ng radio-labeled probe, 100 ng of dIdC, in 25 μ l of binding buffer (50 mM KCl, 1 mM EDTA, 1 mM DTT, 10% glycerol). Samples were incubated for 20 min at room temperature, antibody was added, and the samples incubated 20 min further.

On the basis of these data, we propose the following model. In normal colonic epithelium hTcf-4 is the only expressed member of the Tcf family. The interaction of β -catenin with hTcf-4 is regulated by APC. When appropriate extracellular signals are delivered to an epithelial cell, β -catenin accumulates in a form that is not complexed with GSK3 β -APC, and that enables its nuclear transport and association with hTcf-4. The HMG domain of hTcf-4 binds in a sequence-specific fashion to the regulatory sequences of specific target genes; β -catenin supplies a transactivation domain. Thus, transcriptional activation of target genes occurs only when hTcf-4 is associated with β -catenin. The hTcf-4 target genes remain to be identified. However, the link with APC and catenin suggests that these genes may participate in the generation and turnover of epithelial cells. Upon loss of wild-type APC, monomeric β -catenin accumulates in the absence of extracellular stimuli, leading to uncontrolled transcription of the hTcf-4 target genes. The apparent de novo expression of other members of the Tcf family in some colon carcinoma cell lines might lead to a further deregulation of Tcf target gene expression by the same mechanism. The control of β -catenin-Tcf signaling is likely to be an important part of the gatekeeper function of

APC (19), and its disruption an early step in malignant transformation.

EXAMPLE 5

This example demonstrates that mutant APC protein does not regulate CRT and that a complete set of 20-AA repeats in APC is required to mediate inhibition of CRT.

We tested four APC mutants (FIG. 5A) for their ability to inhibit β -catenin/Tcf-regulated transcription (CRT) in transfection assays. The first mutant, APC331A represents a type of mutation found in the germline of Familial Adenomatous Polyposis (FAP) patients as well as in sporadic tumors (15). The APC331A protein is truncated at codon 331, amino-terminal to the three 15-amino-acid (AA) β -catenin-binding repeats between codons 1020 and 1169. The second mutant, APC1309A, is the most common germline APC mutation (15), a 5-bp deletion that produces a frameshift at codon 1309 and truncation of the protein. The APC1309A protein retains the 15-AA β -catenin repeats but lacks the seven 20-AA repeats between codons 1323 and 2075 that have been implicated in binding and phosphorylation of β -catenin (18). The third mutant, APC1941A, represents one of the most distal somatic mutations observed in colorectal tumors (25). The APC1941A protein is truncated at codon 1941 and therefore contains the 15-AA repeats and all but the last two 20-AA repeats. Finally, APC2644A represents a germline mutation resulting from a 4-bp deletion in codon 2644. Patients with this type of unusual carboxyl-terminal mutation develop few polyps (attenuated polyposis) but have pronounced extracolonic disease, particularly desmoid tumors (26).

Each of the APC mutants was cotransfected with a CRT reporter into the SW480 colorectal cancer cell line. SW480 cells have truncated APC and constitutively active CRT which can be suppressed by exogenous WT APC. Although all four mutants produced comparable levels of APC protein after transfection, they varied in their CRT inhibitory activity. The three mutants found in patients with typical polyposis or cancer were markedly deficient in inhibition of CRT (FIG. 5B). The reduced activity of APC1309A and APC1941A suggests that β -catenin binding is not sufficient for APC-mediated inhibition of CRT and that the complete set of 20-AA repeats is required. Interestingly, the inhibitory activity of the APC2644A mutant associated with attenuated polyposis was comparable to that of WT APC (FIG. 5B), suggesting that the DLG-binding domain at the carboxyl-terminus of APC is not required for down-regulation of CRT.

WT and mutant APC constructs (2 μ g) were transfected into 293, SW480, and HCT116 cells using Lipofectamine (GIBCO/BRL, Gaithersburg). Protein was harvested 24 hours later and subjected to immunoblot analysis with APC monoclonal antibody 1E9 (23). In HCT116 and 293 cells, exogenous WT APC comigrated with the endogenous APC. In SW480 cells, APC1309A comigrated with the endogenous mutant APC. In all other cases, the nonfunctional APC constructs (APC331A, APC 1309A, and APC1941A) produced as much or more protein than the CRT-functional forms of APC (APC WT and APC 2644A).

EXAMPLE 6

This example demonstrates that other components of the APC-regulatory pathway are affected in some cancer cells.

We evaluated CRT in two colorectal tumor cell lines (HCT116 and SW48) that express full-length APC (FIG. 6A). Both HCT116 and SW48 displayed constitutively active CRT and, in contrast to cell lines with truncated APC

(DLD1 and SW480), this activity was not inhibited by exogenous WT APC (FIG. 5B, 6B). Other (noncolorectal cancer) cell lines expressing WT APC do not display constitutive CRT activity. These transfection results suggested that the constitutive CRT in HCT116 and SW48 might be due to an altered downstream component of the APC tumor suppressor pathway.

EXAMPLE 7

This example demonstrates a defect in the gene encoding β -catenin in some cancer cells, which affects CRT.

We evaluated the status of a likely candidate for a downstream component of the APC tumor suppressor pathway, β -catenin, in the same four lines. All four lines expressed similar amounts of apparently intact β -catenin, as assessed by immunoblots (FIG. 7A). However, sequence analysis revealed that both HCT116 and SW48 harbored mutations in the β -catenin gene (CTNNB1) (FIG. 7B). HCT116 had a 3-bp deletion that removed one AA (Ser-45); and SW48 had a C to A missense mutation that changed Ser-33 to Tyr. Analysis of paraffin-embedded archival tissue from the HCT116 patient confirmed the somatic nature of this mutation and its presence in the primary tumor prior to culture. Interestingly, both mutations affected serines that have been implicated in the downregulation of β -catenin through phosphorylation by the $\text{ZW3/GSK3}\beta$ kinase in Xenopus embryos (FIG. 7C) (27,28).

Genomic DNA was isolated from paraffin-embedded normal and tumor tissue from the patient from whom the HCT116 cell line was derived. A 95 bp PCR product encompassing the mutation was then amplified by PCR and directly sequenced using THERMOSEQUENASE (Amersham). The 3 bp deletion was observed in tumor but not in normal tissue.

To test the generality of this mutational mechanism, we evaluated five primary colorectal cancers in which sequencing of the entire coding region of APC revealed no mutations (25). Three of these five tumors were found to contain CTNNB1 mutations (S45F, S45F, and T44A) that altered potential $\text{ZW3/GSK3}\beta$ phosphorylation sites (FIG. 7C). Each mutation appeared to affect only one of the two CTNNB1 alleles and to be somatic.

Genomic DNA was isolated from frozen-sectioned colorectal cancers and a 1001 bp PCR product containing exon 3 of CTNNB1 was then amplified by PCR and directly sequenced using ThermoSequenase (Amersham). An ΔCC change at codon 41 (T41A) and a TCT to TTT at codon 45 (S45F) was observed in one and two tumors, respectively.

EXAMPLE 8

This example demonstrates dominant mutations of CTNNB1 that render CRT insensitive to the effects of WT APC.

Because the β -catenin mutations were heterozygous, we hypothesized that the mutations might exert a dominant effect, rendering a fraction of cellular β -catenin insensitive to APC-mediated down regulation. To test this notion, we performed gel shift analyses with nuclear extracts from untransfected HCT116 cells. In contrast to noncolorectal cancer cell lines with intact APC, HCT116 cells contained a β -catenin/Tcf complex that gel-shifted an optimized Tcf-binding oligonucleotide, and this complex supershifted with anti- β -catenin (FIG. 8A). We also constructed β -catenin expression vectors and compared the biologic activity of the

mutant β -catenin from HCT116 (β -Cat $\Delta 45$) and SW48 (β -Cat S33Y) with that of their WT counterpart. For these experiments, we used the 293 kidney epithelial cell line as it is highly transfectable, exhibits low endogenous CRT, and contains a high level of endogenous APC (FIG. 6A). In the presence of endogenous APC, both mutant β -catenins were at least 6-fold more active than the WT protein and this activity was inhibited by dominant-negative hTcf-4 (FIG. 8B).

Together, these results indicate that disruption of APC-mediated regulation of CRT is critical for colorectal tumorigenesis. This is most commonly achieved by recessive inactivating mutations of both APC alleles but, as shown here, can also be achieved by dominant mutations of CTNNB1 that render CRT insensitive to the effects of WT APC. Our results suggest that APC inhibition of CRT requires phosphorylation of β -catenin at multiple sites. These potential phosphorylation sites are consistent with the known specificity of $\text{ZW3}\beta$ SK3P (29) a serine kinase that negatively regulates β -catenin in *Xenopus* and *Drosophila* cells (27) and that interacts with APC and β -catenin in mammalian cells (23). These results also suggest a functional basis for the occasional CTNNB1 mutations observed in other tumor types (30) and illustrate how a critical pathway in human disease can be illuminated by the discovery of mutations in different components of the pathway. The next step in understanding APC function will be the identification of the genes that are activated by hTcf-4/ β -catenin complexes and inhibited by WT APC. These genes are likely to be related to APC's ability to induce apoptosis in colorectal cancer cells (31).

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(11) NUMBER OF SEQUENCES: 11

(2) INFORMATION FOR SEQ ID NO:1:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2040 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(2) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GGAAAGAAAG	AGAAAGAGAA	AAAGGACAA	CAGCCGGGAG	AGACCAATGG	1260
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CGGCCCTCTG	TCCCAAGCGG	GGCCTGGAC	TGCCCCGAGC	CGCTTTCGAG	1560
CCTCCTCATC	AATTGCACAG	CCGTCGACTT	CTTGGTTACA	TTCCACAGAC	1620
GGACCCAGCC	CCAGCCGCTG	TGCTGCTGCA	CCAAGTCTTT	GAATAGCTGT	1680
AACCCGCGTG	CTTTGTTTTT	GGTTTTGTTT	CACTTTTCTT	AATTTGCCCC	1740
CTTGAAAGGT	TTTGTTTTTT	ACTCTCTTAA	TTTTGTGCCA	TGTGGCTACA	1800
TTATCGAGT	TCAITGGTCA	ATATTTGACC	CATTCTTATT	TCAATTTCTC	1860
TGTAGATGAG	AGAAAGAACCT	CATGATTGGT	ACCAAAATTT	TATCAACAG	1920
TCTTTGTAGC	GTTTAAAAAA	TATATATATA	TACATAACTG	TATGTAGTT	1980
AGTTTTAAAA	GACTGATTAA	AAAACAAAAA	AAAAAAAGC	TTCGAGGGA	2040

(2) INFORMATION FOR SEQ ID NO:2:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 2644 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(3) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGTTTTTTTT	TTTTACCCCC	CTTTTTTATT	TATTAATTTT	TTGCACATTG	AGCGGATCCT	60
TGGGAACGAG	AGAAAAAAGA	AAACCAAACT	CACGCGTGCA	GAAAGATCTC	CCCCCTTCC	120
CCTCCCTTCC	TCCCTCTTTT	CCCCTCCCA	GGAGAAAAAG	ACCCCAAGC	AGAAAAAGT	180
TCACCTTGGG	CTCGTCTTTT	TCTTGCAATA	TTTTTTGGGG	GGGCAAAACT	TGAGGGGGT	240
GATTTTTTTT	GGCTTTTCTT	CCTCCTTCAT	TTTTCTTCCA	AAATTTGCTG	TGGTGGGTGA	300
AAAAAAAATG	CCGACAGCTG	ACGGCGGTGG	AGGGGATGAC	CTAGGCGCCA	ACAGCGAACT	360
GATTTCTCTT	AAAGACGAGG	GGGAAACAGG	GGAGAAAGAG	TCCGAAAACCT	CCTCGGCGAG	420
GAGGGAATTG	GCTGATGCTA	AATCGTCTCT	AGTCAATGAA	TGAGAAACGA	ATCAAAACAG	480
CTCTCTCGAT	TCCGAGGCGG	AAAGACGGCC	TCCGGCTCGC	TCCGAAAAGT	TCCGAGACAA	540
ATCCCGGGAA	AGTTTGGAAG	AAGCGGCGCA	GAGGCAAGAT	GAGGGGCTCT	TAAAGGGGCG	600
ACCGTATCCC	GGCTACCTCT	TCATCATGAT	CCCCGACCTG	ACGAGCCCTT	ACCTCCCTCA	660
GCGATCCGTC	TGCGGACACG	CCGGAACCTA	TCTCTAGATG	AAATGGCCAC	TGCTTGATGT	720
CCAGGCAAGG	AGCCTCCAGA	GTAGACAAGC	CCTCAAGGAT	GCCCGGTCCC	CATCACCGGC	780
ACACATTGTC	TCTAACAAAG	TGCCAGTGGT	GCAGCACCTT	CACCATGTCC	ACCCCTCTAC	840
GCCTCTTATC	ACGTACAGCA	ATGAACACTT	CACGCCGGGA	AAACCACTCT	CACACTTACC	900

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AGCCGACGTA GACCCCAAAA CAGGAATCCC ACGGCCTCCG CACCCCTCAG ATATATCCCC 960
GTATTACCCA CTATCGCCTG GCACCGTAGG ACAAAATCCCC CATCCGCTAG GATGGTTAGT 1020
ACCCACAGCAA GGTCAACACG TGTACCCAAAT CACGACAGGA GGATTCAGAC ACCCCTACCC 1080
CACAGCTCTG ACCGTCAATG CTTCCGTGTC CAGGTTCCCT CCCCATATGG TCCACCACCA 1140
TCATACGCTA CACACGACGG GCATTCGCGA TCCGGCCATA GTACACACCA CAGTCAAAACA 1200
GGAATCGTCC CAGAAGTATG TCGGCTCACT CCATAGTTCA AAGCATCAGG ACTCCAAAAA 1260
GGAAGAAAGAA AAGAAGAAAG CCACATAAAA GAAACCTCTT AATGCATTCA TGTTGTATAT 1320
GAAAGAAATG AGAGCAAAAG TCGTAGCTGA GTGCACGTTG AAAGAAAGCG CGGCCATCAA 1380
CCAGATCCTT GGGCGGAGGT GGCATGCACT GTCCAGAGAA GAGCAAGCGA AATACTACGA 1440
GCTGCCCGGG AAGGAGCGAC AGCTTCATAT GCAACTGTAT CCGGCTGGT CCGCGCGGGA 1500
TAACATATGA AAGAAGAAAG AGAAGAAAAA GACAAAGCAG CCGGAGAGA CCAATGAACA 1560
CAGCGAATGT TTCTTAAATC CTTGCCCTTC ACTTCCTCCG ATTACAGACC GTTACGCTCC 1620
TAAGAAATGC CAGCGCGCTT TTGGCCTTGA TCAACAGAAAT AACTGGTGGC GCCCTTGCA 1680
GAGAAAAAAA AAGTGCCTTC GCTACATACA AGGTGAAAGC AGCTGCCCTA GCCACCCCTC 1740
TTCAGATGGA AGCTTACTAG ATTCCGCTCC CCCCCTCCCC AACCTGCTAG GCTCCCTCC 1800
CCGAGACGCC AAGTCACAGA CTGAGCAGAC CCAAGCTCTG TCGCTGCTCC TGAAGCCCGA 1860
CCCCCTGGCC CACCTGTCCA TGATGCTTCC GCCACCCGCC CTCCTGCTCG CTGAGGCCAC 1920
CCACAAAGCC TCCGCTCTCT GTCCCAACGG GGCCTGTGAC CTGCCCCCA 1980
GCCTGCCGCC CCGCTCTCAT CAATTGCACA GCCGTGCACT TCTTGGTTAC ATTCCCAAG 2040
CTCCCTGGCC GGACCCAGC CCAAGCCGCT GTGCTGCTGC ACCAAGCTT TAGAATAGCT 2100
TTAGCGTCGT GAACCCCGCT GCTTTGTTTA TGGTTTTGTT TCACTTTTCT TAATTTGCC 2160
CCCACCCCCA CTTTGAAGAG TTTTGTTTTG TACTCTCTTA ATTTGTGCC ATGTGGCTAC 2220
ATTAGTTGAT GTTTATCGAG TTCATTGGTC AATATTTGAC CCATTCCTAT TTCAATTTCT 2280
CTTTTAAAT ATGTAGATGA GAGAAAGAAC TCATGATTGG TACCAAAAT TTTATCAACA 2340
GCTGTTTAAA GTCTTTGTAG CGTTTAAAAA ATATATATAT ATACATAACT GTTATGTAGT 2400
TCGGATAGCT TAGTTTTAAA AGACTGATTA AAAAAACAAA AAAA 2444

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(2) INFORMATION FOR SEQ ID NO:3:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCCTTTGATC TTACC

15

(2) INFORMATION FOR SEQ ID NO:4:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(* 1) MOLECULE TYPE: cDNA

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCCTTTGGCC TTACC

15

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(2) INFORMATION FOR SEQ ID NO:5:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 442 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Nasal

(a) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```

Met  Pro  Glu  Leu  Asn  Gly  Gly  Gly  Gly  Asp  Asp  Leu  Gly  Ala  Asn  Asp
 1      5      10      15

Glu  Leu  Ile  Ser  Phe  Lys  Asp  Glu  Gly  Glu  Glu  Glu  Lys  Ser  Ser
20     25     30

Glu  Asn  Ser  Ser  Ala  Glu  Arg  Asp  Leu  Ala  Asp  Val  Lys  Ser  Ser  Leu
35     40     45

Val  Asn  Glu  Ser  Glu  Thr  Asn  Glu  Asn  Ser  Ser  Ser  Asp  Ser  Glu  Ala
50     55     60

Glu  Arg  Arg  Pro  Pro  Pro  Arg  Ser  Glu  Ser  Phe  Arg  Asp  Lys  Ser  Arg
65     70     75     80

Glu  Ser  Leu  Glu  Glu  Ala  Ala  Lys  Arg  Glu  Asn  Gly  Gly  Leu  Phe  Lys
85     90     95

Gly  Pro  Pro  Tyr  Pro  Gly  Tyr  Pro  Phe  Ile  Met  Ile  Pro  Asn  Leu  Thr
100    105    110

Ser  Pro  Tyr  Leu  Pro  Lys  Arg  Ser  Val  Ser  Pro  Thr  Ala  Arg  Thr  Tyr
115    120    125

Leu  Glu  Met  Lys  Trp  Pro  Leu  Leu  Asp  Val  Glu  Ala  Gly  Ser  Leu  Glu
130    135    140

Ser  Arg  Glu  Ala  Leu  Lys  Asp  Ala  Arg  Ser  Pro  Ser  Pro  Ala  His  Ile
145    150    155    160

Val  Ser  Asn  Lys  Val  Pro  Val  Val  Glu  His  Pro  His  His  Val  His  Pro
165    170    175

Leu  Thr  Pro  Leu  Ile  Thr  Tyr  Ser  Asn  Glu  His  Phe  Thr  Pro  Gly  Asn
180    185    190

Pro  Pro  Pro  His  Leu  Pro  Ala  Asp  Val  Asp  Pro  Lys  Thr  Gly  Ile  Pro
195    200    205

Arg  Pro  Pro  His  Pro  Pro  Asp  Ile  Ser  Pro  Tyr  Tyr  Pro  Leu  Ser  Pro
210    215    220

Gly  Thr  Val  Gly  Glu  Ile  Pro  His  Pro  Leu  Gly  Trp  Leu  Val  Pro  Glu
225    230    235    240

Glu  Gly  Glu  Pro  Val  Tyr  Pro  Ile  Thr  Thr  Gly  Gly  Phe  Arg  His  Pro
245    250    255

Tyr  Pro  Thr  Ala  Leu  Thr  Val  Asn  Ala  Ser  Val  Ser  Arg  Phe  Pro  Pro
260    265    270

His  Met  Val  Pro  Pro  His  His  Thr  Leu  His  Thr  Thr  Gly  Ile  Pro  His
275    280    285

Pro  Ala  Ile  Val  Thr  Pro  Thr  Val  Lys  Glu  Glu  Ser  Ser  Glu  Ser  Asp
290    295    300

Val  Gly  Ser  Leu  His  Ser  Ser  Lys  His  Glu  Asp  Ser  Lys  Lys  Glu  Glu
305    310    315

Glu  Lys  Lys  Lys  Pro  His  Ile  Lys  Lys  Pro  Leu  Asn  Ala  Phe  Met  Leu
325    330    335

Tyr  Met  Lys  Glu  Met  Arg  Ala  Lys  Val  Val  Ala  Glu  Cys  Thr  Leu  Lys
340    345    350

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Glu	Ser	Ala	Ala	Ile	Asn	Gln	Ile	Leu	Gly	Arg	Arg	Trp	His	Ala	Leu	
		355					360					365				
Ser	Arg	Glu	Glu	Gln	Ala	Lys	Tyr	Tyr	Gln	Leu	Ala	Arg	Lys	Gln	Arg	
		370				375					380					
Gln	Leu	His	Met	Gln	Leu	Tyr	Pro	Gly	Trp	Ser	Ala	Arg	Asp	Asn	Tyr	
		385			390					395					400	
Gly	Lys	Lys	Lys	Lys	Arg	Lys	Arg	Asp	Lys	Gln	Pro	Gly	Glu	Thr	Asn	
				405					410					415		
Gly	Glu	Lys	Lys	Ser	Ala	Phe	Ala	Thr	Tyr	Lys	Val	Lys	Ala	Ala	Ala	
				420				425					430			
Ser	Ala	His	Pro	Leu	Gln	Met	Glu	Ala	Tyr							
		435					440									

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 596 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met	Pro	Gln	Leu	Asn	Gly	Gly	Gly	Asp	Asp	Leu	Gly	Ala	Asn	Asp		
1				5				10					15			
Gln	Leu	Ile	Ser	Phe	Lys	Asp	Gln	Gly	Gln	Gln	Gln	Lys	Ser	Ser		
		20					25					30				
Glu	Asn	Ser	Ser	Ala	Gln	Arg	Asp	Leu	Ala	Asp	Val	Lys	Ser	Ser	Leu	
		35				40						45				
Val	Asn	Gln	Ser	Glu	Thr	Asn	Gln	Asn	Ser	Ser	Ser	Asp	Ser	Glu	Ala	
		50			55						60					
Glu	Arg	Arg	Pro	Pro	Pro	Arg	Ser	Glu	Ser	Phe	Arg	Asp	Lys	Ser	Arg	
	65				70					75					80	
Glu	Ser	Leu	Gln	Gln	Ala	Ala	Lys	Arg	Gln	Asp	Gly	Gly	Leu	Phe	Lys	
			85						90				95			
Gly	Pro	Pro	Tyr	Pro	Gly	Tyr	Pro	Phe	Ile	Met	Ile	Pro	Asp	Leu	Thr	
			100					105					110			
Ser	Pro	Tyr	Leu	Pro	Asn	Gly	Ser	Val	Ser	Pro	Thr	Ala	Arg	Thr	Tyr	
		115				120					125					
Leu	Gln	Met	Lys	Trp	Pro	Leu	Leu	Asp	Val	Gln	Ala	Gly	Ser	Leu	Gln	
		130			135						140					
Ser	Arg	Gln	Ala	Leu	Lys	Asp	Ala	Arg	Ser	Pro	Ser	Pro	Ala	His	Ile	
		145			150				155					160		
Val	Ser	Asn	Lys	Val	Pro	Val	Val	Gln	His	Pro	His	His	Val	His	Pro	
			165					170					175			
Leu	Thr	Pro	Leu	Ile	Thr	Tyr	Ser	Asn	Glu	His	Phe	Thr	Pro	Gly	Asn	
		180					185						190			
Pro	Pro	Pro	His	Leu	Pro	Ala	Asp	Val	Asp	Pro	Lys	Thr	Gly	Ile	Pro	
		195				200						205				
Arg	Pro	Pro	His	Pro	Pro	Asp	Ile	Ser	Pro	Tyr	Tyr	Pro	Leu	Ser	Pro	
		210				215				220						
Gly	Thr	Val	Gly	Gln	Ile	Pro	His	Pro	Leu	Gly	Trp	Leu	Val	Pro	Gln	
		225			230					235				240		
Gln	Gly	Gln	Pro	Val	Tyr	Pro	Ile	Thr	Thr	Gly	Gly	Phe	Arg	His	Pro	
			245					250					255			
Tyr	Pro	Thr	Ala	Leu	Thr	Val	Asn	Ala	Ser	Val	Ser	Arg	Phe	Pro	Pro	

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260										265										270									
His	Met	Val	Pro	Pro	His	His	Thr	Leu	His	Thr	Thr	Gly	Ile	Pro	His														
		275																											
Pro	Ala	Ile	Val	Thr	Pro	Thr	Val	Lys	Gln	Glu	Ser	Ser	Gln	Ser	Asp														
		290						295						300															
Val	Gly	Ser	Leu	His	Ser	Ser	Lys	His	Gln	Asp	Ser	Lys	Lys	Glu	Glu														
		305						310						315															
Glu	Lys	Lys	Lys	Pro	His	Ile	Lys	Lys	Pro	Leu	Asn	Ala	Phe	Met	Leu														
								325						330															
Tyr	Met	Lys	Gln	Met	Arg	Ala	Lys	Val	Val	Ala	Glu	Cys	Thr	Leu	Lys														
								340						345															
Glu	Ser	Ala	Ala	Ile	Asn	Gln	Ile	Leu	Gly	Arg	Arg	Trp	His	Ala	Leu														
								360						365															
Ser	Arg	Gln	Glu	Gln	Ala	Lys	Tyr	Tyr	Gln	Leu	Ala	Arg	Lys	Glu	Arg														
								375						380															
Gln	Leu	His	Met	Gln	Leu	Tyr	Pro	Gly	Trp	Ser	Ala	Arg	Asp	Asn	Tyr														
								390						395															
Gly	Lys	Lys	Lys	Lys	Arg	Lys	Arg	Asp	Lys	Gln	Pro	Gly	Glu	Thr	Asn														
								405						410															
Glu	His	Ser	Glu	Cys	Phe	Leu	Asn	Pro	Cys	Leu	Ser	Leu	Pro	Ile															
								420						425															
Thr	Asp	Leu	Ser	Ala	Pro	Lys	Lys	Cys	Arg	Ala	Arg	Phe	Gly	Leu	Asp														
								440						445															
Gln	Gln	Asn	Asn	Trp	Cys	Gly	Pro	Cys	Arg	Arg	Lys	Lys	Lys	Cys	Val														
								455						460															
Arg	Tyr	Ile	Gln	Gly	Glu	Gly	Ser	Cys	Leu	Ser	Pro	Pro	Ser	Ser	Asp														
								470						475															
Gly	Ser	Leu	Leu	Asp	Ser	Pro	Pro	Pro	Ser	Pro	Asn	Leu	Leu	Gly	Ser														
								485						490															
Pro	Pro	Arg	Asp	Ala	Lys	Ser	Gln	Thr	Gln	Gln	Thr	Gln	Pro	Leu	Ser														
								500						505															
Leu	Ser	Leu	Lys	Pro	Asp	Pro	Leu	Ala	His	Leu	Ser	Met	Met	Pro	Pro														
								515						520															
Pro	Pro	Ala	Leu	Leu	Leu	Ala	Gln	Ala	Thr	His	Lys	Ala	Ser	Ala	Leu														
								535						540															
Cys	Pro	Asn	Gly	Ala	Leu	Asp	Leu	Pro	Pro	Ala	Ala	Leu	Gln	Pro	Ala														
								550						555															
Ala	Pro	Ser	Ser	Ser	Ile	Ala	Gln	Pro	Ser	Thr	Ser	Trp	Leu	His	Ser														
								565						570															
His	Ser	Ser	Leu	Ala	Gly	Thr	Gln	Pro	Gln	Pro	Leu	Ser	Leu	Val	Thr														
								580						585															
Lys	Ser	Leu	Gln																										
								595																					

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2973 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Met Ala Ala Ala Ser Tyr Asp Gln Leu Leu Lys Gln Val Gln Ala Leu
 1 5 10 15

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Lys Met Glu Asn Ser Asn Leu Arg Gln Gln Leu Glu Asp Asn Ser Asn
 20 25 30
 His Leu Thr Lys Leu Glu Thr Glu Ala Ser Asn Met Lys Glu Val Leu
 35 40 45
 Lys Gln Leu Gln Gly Ser Ile Glu Asp Glu Ala Met Ala Ser Ser Gly
 50 55 60
 Gln Ile Asp Leu Leu Glu Arg Leu Lys Glu Leu Asn Leu Asp Ser Ser
 65 70 75 80
 Asn Phe Pro Gly Val Lys Leu Arg Ser Lys Met Ser Leu Arg Ser Tyr
 85 90 95
 Gly Ser Arg Gln Gly Ser Val Ser Ser Arg Ser Gly Glu Cys Ser Pro
 100 105 110
 Val Pro Met Gly Ser Phe Pro Arg Arg Gly Phe Val Asn Gly Ser Arg
 115 120 125
 Glu Ser Thr Gly Tyr Leu Glu Glu Leu Glu Lys Glu Arg Ser Leu Leu
 130 135 140
 Leu Ala Asp Leu Asp Lys Glu Gln Lys Glu Lys Asp Trp Tyr Tyr Ala
 145 150 155 160
 Glu Leu Gln Asn Leu Thr Lys Arg Ile Asp Ser Leu Pro Leu Thr Glu
 165 170 175
 Asn Phe Ser Leu Gln Thr Asp Met Thr Arg Arg Gln Leu Glu Tyr Glu
 180 185 190
 Ala Arg Glu Ile Arg Val Ala Met Gln Gln Gln Leu Gly Thr Cys Gln
 195 200 205
 Asp Met Glu Lys Arg Ala Gln Arg Arg Ile Ala Arg Ile Gln Gln Ile
 210 215 220
 Glu Lys Asp Ile Leu Arg Ile Arg Gln Leu Leu Gln Ser Gln Ala Thr
 225 230 235 240
 Glu Ala Glu Arg Ser Ser Gln Asn Lys His Glu Thr Gly Ser His Asp
 245 250 255
 Ala Glu Arg Gln Asn Glu Gly Gln Gly Val Gly Glu Ile Asn Met Ala
 260 265 270
 Thr Ser Gly Asn Gly Gln Gly Ser Thr Thr Arg Met Asp His Glu Thr
 275 280 285
 Ala Ser Val Leu Ser Ser Ser Ser Thr His Ser Ala Pro Arg Arg Leu
 290 295 300
 Thr Ser His Leu Gly Thr Lys Val Glu Met Val Tyr Ser Leu Leu Ser
 305 310 315 320
 Met Leu Gly Thr His Asp Lys Asp Asp Met Ser Arg Thr Leu Leu Ala
 325 330 335
 Met Ser Ser Ser Gln Asp Ser Cys Ile Ser Met Arg Gln Ser Gly Cys
 340 345 350
 Leu Pro Leu Leu Ile Gln Leu Leu His Gly Asn Asp Lys Asp Ser Val
 355 360 365
 Leu Leu Gly Asn Ser Arg Gly Ser Lys Glu Ala Arg Ala Arg Ala Ser
 370 375 380
 Ala Ala Leu His Asn Ile Ile His Ser Gln Pro Asp Asp Lys Arg Gly
 385 390 395 400
 Arg Arg Glu Ile Arg Val Leu His Leu Leu Gln Gln Ile Arg Ala Tyr
 405 410 415
 Cys Glu Thr Cys Trp Glu Trp Gln Gln Ala His Gln Pro Gly Met Asp
 420 425 430
 Gln Asp Lys Asn Pro Met Pro Ala Pro Val Glu His Gln Ile Cys Pro

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433					440					445					
Ala	Val	Cys	Val	Leu	Met	Lys	Leu	Ser	Phe	Asp	Glu	Glu	His	Arg	His
450						455					460				
Ala	Met	Asn	Glu	Leu	Gly	Gly	Leu	Gln	Ala	Ile	Ala	Glu	Leu	Leu	Gln
465					470					475					480
Val	Asp	Cys	Glu	Met	Tyr	Gly	Leu	Thr	Asn	Asp	His	Tyr	Ser	Ile	Thr
				485					490					495	
Leu	Arg	Arg	Tyr	Ala	Gly	Met	Ala	Leu	Thr	Asn	Leu	Thr	Phe	Gly	Asp
			500				505						510		
Val	Ala	Asn	Lys	Ala	Thr	Leu	Cys	Ser	Met	Lys	Gly	Cys	Met	Arg	Ala
		515					520					525			
Leu	Val	Ala	Gln	Leu	Lys	Ser	Glu	Ser	Glu	Asp	Leu	Gln	Gln	Val	Ile
		530				535					540				
Ala	Ser	Val	Leu	Arg	Asn	Leu	Ser	Trp	Arg	Ala	Asp	Val	Asn	Ser	Lys
545					550					555					560
Lys	Thr	Leu	Arg	Glu	Val	Gly	Ser	Val	Lys	Ala	Leu	Met	Glu	Cys	Ala
			565						570					575	
Leu	Glu	Val	Lys	Lys	Glu	Ser	Thr	Leu	Lys	Ser	Val	Leu	Ser	Ala	Leu
		580						585					590		
Trp	Asn	Leu	Ser	Ala	His	Cys	Thr	Glu	Asn	Lys	Ala	Asp	Ile	Cys	Ala
		595					600						605		
Val	Asp	Gly	Ala	Leu	Ala	Phe	Leu	Val	Gly	Thr	Leu	Thr	Tyr	Arg	Ser
		610				615					620				
Gln	Thr	Asn	Thr	Leu	Ala	Ile	Ile	Glu	Ser	Gly	Gly	Gly	Ile	Leu	Arg
625					630					635					640
Asn	Val	Ser	Ser	Leu	Ile	Ala	Thr	Asn	Glu	Asp	His	Arg	Gln	Ile	Leu
			645						650					655	
Arg	Glu	Asn	Asn	Cys	Leu	Gln	Thr	Leu	Leu	Gln	His	Leu	Lys	Ser	His
			660					665					670		
Ser	Leu	Thr	Ile	Val	Ser	Asn	Ala	Cys	Gly	Thr	Leu	Trp	Asn	Leu	Ser
			675				680						685		
Ala	Arg	Asn	Pro	Lys	Asp	Gln	Glu	Ala	Leu	Trp	Asp	Met	Gly	Ala	Val
		690				695					700				
Ser	Met	Leu	Lys	Asn	Leu	Ile	His	Ser	Lys	His	Lys	Met	Ile	Ala	Met
705					710					715					720
Gly	Ser	Ala	Ala	Ala	Leu	Arg	Asn	Leu	Met	Ala	Asn	Arg	Pro	Ala	Lys
			725						730					735	
Tyr	Lys	Asp	Ala	Asn	Ile	Met	Ser	Pro	Gly	Ser	Ser	Leu	Pro	Ser	Leu
			740				745						750		
His	Val	Arg	Lys	Gln	Lys	Ala	Leu	Glu	Ala	Glu	Leu	Asp	Ala	Gln	His
		755					760						765		
Leu	Ser	Glu	Thr	Phe	Asp	Asn	Ile	Asp	Asn	Leu	Ser	Pro	Lys	Ala	Ser
			770			775						780			
His	Arg	Ser	Lys	Gln	Arg	His	Lys	Gln	Ser	Leu	Tyr	Gly	Asp	Tyr	Val
785					790					795					800
Phe	Asp	Thr	Asn	Arg	His	Asp	Asp	Asn	Arg	Ser	Asp	Asn	Phe	Asn	Thr
			805						810				815		
Gly	Asn	Met	Thr	Val	Leu	Ser	Pro	Tyr	Leu	Asn	Thr	Thr	Val	Leu	Pro
			820				825						830		
Ser	Ser	Ser	Ser	Ser	Arg	Gly	Ser	Leu	Asp	Ser	Ser	Arg	Ser	Glu	Lys
			835				840						845		
Asp	Arg	Ser	Leu	Glu	Arg	Gln	Arg	Gly	Ile	Gly	Leu	Gly	Asn	Tyr	His
850						855						860			

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Pro	Ala	Thr	Glu	Asn	865	Pro	Gly	Thr	Ser	Ser	870	Lys	Arg	Gly	Leu	Gln	Ile	880
Ser	Thr	Thr	Ala	Ala	885	Gln	Ile	Ala	Lys	Val	Met	Glu	Glu	Val	Ser	Ala		
Ile	His	Thr	Ser	Gln	900	Glu	Asp	Arg	Ser	Ser	Gly	Ser	Thr	Thr	Glu	Leu		
His	Cys	Val	Thr	Asp	915	Gln	Arg	Asn	Ala	Leu	Arg	Arg	Ser	Ser	Ala	Ala		
His	Thr	His	Ser	Asn	930	Thr	Tyr	Asn	Phe	Thr	Lys	Ser	Glu	Asn	Ser	Asn		
Arg	Thr	Cys	Ser	Met	945	Pro	Tyr	Ala	Lys	Leu	Glu	Tyr	Lys	Arg	Ser	Ser		
Asn	Asp	Ser	Leu	Asn	965	Ser	Val	Ser	Ser	Ser	Asp	Gly	Tyr	Gly	Lys	Arg		
Gly	Gln	Met	Lys	Pro	980	Ser	Ile	Glu	Ser	Tyr	Ser	Glu	Asp	Asp	Glu	Ser		
Lys	Phe	Cys	Ser	Tyr	995	Gly	Gln	Tyr	Pro	Ala	Asp	Leu	Ala	His	Lys	Ile		
His	Ser	Ala	Asn	His	1010	Met	Asp	Asp	Asn	Asp	Gly	Glu	Leu	Asp	Thr	Pro		
Ile	Asn	Tyr	Ser	Leu	1025	Lys	Tyr	Ser	Asp	Glu	Gln	Leu	Asn	Ser	Gly	Arg		
Gln	Ser	Pro	Ser	Gln	1045	Asn	Glu	Arg	Trp	Ala	Arg	Pro	Lys	His	Ile	Ile		
Glu	Asp	Glu	Ile	Lys	1060	Gln	Ser	Glu	Gln	Arg	Gln	Ser	Arg	Asn	Gln	Ser		
Thr	Thr	Tyr	Pro	Val	1075	Tyr	Thr	Glu	Ser	Thr	Asp	Asp	Lys	His	Leu	Lys		
Phe	Gln	Pro	His	Phe	1090	Gly	Gln	Glu	Cys	Val	Ser	Pro	Tyr	Arg	Ser			
Arg	Gly	Ala	Asn	Gly	1105	Ser	Glu	Thr	Asn	Arg	Val	Gly	Ser	Asn	His	Gly		
Ile	Asn	Gln	Asa	Val	1125	Ser	Gln	Ser	Leu	Cys	Gln	Gln	Asp	Asp	Tyr	Glu		
Asp	Asp	Lys	Pro	Thr	1140	Asn	Tyr	Ser	Glu	Arg	Tyr	Ser	Glu	Glu	Glu	Gln		
His	Glu	Glu	Glu	Glu	1155	Arg	Pro	Thr	Asn	Tyr	Ser	Ile	Lys	Tyr	Asn	Glu		
Glu	Lys	Arg	His	Val	1170	Asp	Gln	Pro	Ile	Asp	Tyr	Ser	Leu	Lys	Tyr	Ala		
Thr	Asp	Ile	Pro	Ser	1185	Ser	Gln	Lys	Gln	Ser	Phe	Ser	Phe	Ser	Lys	Ser		
Ser	Ser	Gly	Gln	Ser	1205	Ser	Lys	Thr	Glu	His	Met	Ser	Ser	Ser	Ser	Glu		
Asn	Thr	Ser	Thr	Pro	1220	Ser	Ser	Asn	Ala	Lys	Arg	Gln	Asn	Gln	Leu	His		
Pro	Ser	Ser	Ala	Gln	1235	Ser	Arg	Ser	Gly	Gln	Pro	Gln	Lys	Ala	Ala	Thr		
Cys	Lys	Val	Ser	Ser	1250	Ile	Asn	Gln	Gln	Thr	Ile	Gln	Thr	Tyr	Cys	Val		
Gln	Asp	Thr	Pro	Ile	1265	Cys	Phe	Ser	Arg	Cys	Ser	Ser	Leu	Ser	Ser	Leu		
Ser	Ser	Ala	Glu	Asp	1285	Gln	Ile	Gly	Cys	Asn	Gln	Thr	Thr	Gln	Glu	Ala		

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Asp Ser Ala Asn Thr Leu Glu Ile Ala Glu Ile Lys Glu Lys Ile Gly
 1300 1305 1310
 Thr Arg Ser Ala Glu Asp Pro Val Ser Glu Val Pro Ala Val Ser Glu
 1315 1320 1325
 His Pro Arg Thr Lys Ser Ser Arg Leu Glu Gly Ser Ser Leu Ser Ser
 1330 1335 1340
 Glu Ser Ala Arg His Lys Ala Val Glu Phe Ser Ser Gly Ala Lys Ser
 1345 1350 1355 1360
 Pro Ser Lys Ser Gly Ala Glu Thr Pro Lys Ser Pro Pro Glu His Tyr
 1365 1370 1375
 Val Glu Glu Thr Pro Leu Met Phe Ser Arg Cys Thr Ser Val Ser Ser
 1380 1385 1390
 Leu Asp Ser Phe Glu Ser Arg Ser Ile Ala Ser Ser Val Glu Ser Glu
 1395 1400 1405
 Pro Cys Ser Gly Met Val Ser Gly Ile Ile Ser Pro Ser Asp Leu Pro
 1410 1415 1420
 Asp Ser Pro Gly Glu Thr Met Pro Pro Ser Arg Ser Lys Thr Pro Pro
 1425 1430 1435 1440
 Pro Pro Pro Glu Thr Ala Glu Thr Lys Arg Glu Val Pro Lys Asn Lys
 1445 1450 1455
 Ala Pro Thr Ala Glu Lys Arg Glu Ser Gly Pro Lys Glu Ala Ala Val
 1460 1465 1470
 Asn Ala Ala Val Glu Arg Val Glu Val Leu Pro Asp Ala Asp Thr Leu
 1475 1480 1485
 Leu His Phe Ala Thr Glu Ser Thr Pro Asp Gly Phe Ser Cys Ser Ser
 1490 1495 1500
 Ser Leu Ser Ala Leu Ser Leu Asp Glu Pro Phe Ile Glu Lys Asp Val
 1505 1510 1515 1520
 Glu Leu Arg Ile Met Pro Pro Val Glu Glu Asn Asp Asn Gly Asn Glu
 1525 1530 1535
 Thr Glu Ser Glu Glu Pro Lys Glu Ser Asn Glu Asn Glu Glu Lys Glu
 1540 1545 1550
 Ala Glu Lys Thr Ile Asp Ser Glu Lys Asp Leu Leu Asp Asp Ser Asp
 1555 1560 1565
 Asp Asp Asp Ile Glu Ile Leu Glu Glu Cys Ile Ile Ser Ala Met Pro
 1570 1575 1580
 Thr Lys Ser Ser Arg Lys Ala Lys Lys Pro Ala Glu Thr Ala Ser Lys
 1585 1590 1595 1600
 Leu Pro Pro Pro Val Ala Arg Lys Pro Ser Glu Leu Pro Val Tyr Lys
 1605 1610 1615
 Leu Leu Pro Ser Glu Asn Arg Leu Glu Pro Glu Lys His Val Ser Phe
 1620 1625 1630
 Thr Pro Gly Asp Asp Met Pro Arg Val Tyr Cys Val Glu Gly Thr Pro
 1635 1640 1645
 Ile Asn Phe Ser Thr Ala Thr Ser Leu Ser Asp Leu Thr Ile Glu Ser
 1650 1655 1660
 Pro Pro Asn Glu Leu Ala Ala Gly Glu Gly Val Arg Gly Gly Ala Glu
 1665 1670 1675 1680
 Ser Gly Glu Phe Glu Lys Arg Asp Thr Ile Pro Thr Glu Gly Arg Ser
 1685 1690 1695
 Thr Asp Glu Ala Glu Gly Gly Lys Thr Ser Ser Val Thr Ile Pro Glu
 1700 1705 1710
 Leu Asp Asp Asn Lys Ala Glu Glu Gly Asp Ile Leu Ala Glu Cys Ile

1715					1720					1725				
Asn	Ser	Ala	Met	Pro	Lys	Gly	Lys	Ser	His	Lys	Pro	Phe	Arg	Val
1730					1735					1740				
Lys	Ile	Met	Asp	Gln	Val	Gln	Gln	Ala	Ser	Ala	Ser	Ser	Ser	Ala
1745				1750						1755				1760
Asn	Lys	Asn	Gln	Leu	Asp	Gly	Lys	Lys	Lys	Lys	Pro	Thr	Ser	Pro
				1765					1770					1775
Lys	Pro	Ile	Pro	Gln	Asn	Thr	Glu	Tyr	Arg	Thr	Arg	Val	Arg	Lys
				1780					1785					1790
Ala	Asp	Ser	Lys	Asn	Asn	Leu	Asn	Ala	Glu	Arg	Val	Phe	Ser	Asp
				1795					1800					1805
Lys	Asp	Ser	Lys	Lys	Gln	Asn	Leu	Lys	Asn	Asn	Ser	Lys	Asp	Phe
				1810					1815					1820
Asp	Lys	Leu	Pro	Asn	Asn	Glu	Asp	Arg	Val	Arg	Gly	Ser	Phe	Ala
1825				1830					1835					1840
Asp	Ser	Pro	His	His	Tyr	Thr	Pro	Ile	Glu	Gly	Thr	Pro	Tyr	Cys
				1845					1850					1855
Ser	Arg	Asn	Asp	Ser	Leu	Ser	Ser	Leu	Asp	Phe	Asp	Asp	Asp	Val
				1860					1865					1870
Asp	Leu	Ser	Arg	Glu	Lys	Ala	Glu	Leu	Arg	Lys	Ala	Lys	Glu	Asn
				1875					1880					1885
Glu	Ser	Glu	Ala	Lys	Val	Thr	Ser	His	Thr	Glu	Leu	Thr	Ser	Asn
				1890					1895					1900
Gln	Ser	Ala	Asn	Lys	Thr	Gln	Ala	Ile	Ala	Lys	Gln	Pro	Ile	Asn
				1905					1910					1915
Gly	Gln	Pro	Lys	Pro	Ile	Leu	Gln	Lys	Gln	Ser	Thr	Phe	Pro	Gln
				1920					1925					1930
Ser	Lys	Asp	Ile	Pro	Asp	Arg	Gly	Ala	Ala	Thr	Asp	Glu	Lys	Leu
				1935					1940					1945
Asn	Phe	Ala	Ile	Glu	Asn	Thr	Pro	Val	Cys	Phe	Ser	His	Asn	Ser
				1950					1955					1960
Leu	Ser	Ser	Leu	Ser	Asp	Ile	Asp	Gln	Glu	Asn	Asn	Asn	Lys	Glu
				1965					1970					1975
Glu	Pro	Ile	Lys	Glu	Thr	Glu	Pro	Pro	Asp	Ser	Gln	Gly	Glu	Pro
1985				1990					1995					2000
Lys	Pro	Gln	Ala	Ser	Gly	Tyr	Ala	Pro	Lys	Ser	Phe	His	Val	Glu
				2005					2010					2015
Thr	Pro	Val	Cys	Phe	Ser	Arg	Asn	Ser	Ser	Leu	Ser	Ser	Leu	Ser
				2020					2025					2030
Asp	Ser	Glu	Asp	Asp	Leu	Leu	Gln	Glu	Cys	Ile	Ser	Ser	Ala	Met
				2035					2040					2045
Lys	Lys	Lys	Lys	Pro	Ser	Arg	Leu	Lys	Gly	Asp	Asn	Glu	Lys	His
				2050					2055					2060
Pro	Arg	Asn	Met	Gly	Gly	Ile								

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Gly Ser Pro Phe His Leu Thr Pro Asp Glu Glu Lys Pro Phe Thr	2145	2150	2155	2160
Ser Asn Lys Gly Pro Arg Ile Leu Lys Pro Gly Glu Lys Ser Thr Leu	2165	2170	2175	
Glu Thr Lys Lys Ile Glu Ser Glu Ser Lys Gly Ile Lys Gly Gly Lys	2180	2185	2190	
Lys Val Tyr Lys Ser Leu Ile Thr Gly Lys Val Arg Ser Asn Ser Glu	2195	2200	2205	
Ile Ser Gly Glu Met Lys Glu Pro Leu Glu Ala Asn Met Pro Ser Ile	2210	2215	2220	
Ser Arg Gly Arg Thr Met Ile His Ile Pro Gly Val Arg Asn Ser Ser	2225	2230	2235	2240
Ser Ser Thr Ser Pro Val Ser Lys Lys Gly Pro Pro Leu Lys Thr Pro	2245	2250	2255	
Ala Ser Lys Ser Pro Ser Glu Gly Glu Thr Ala Thr Thr Ser Pro Arg	2260	2265	2270	
Gly Ala Lys Pro Ser Val Lys Ser Glu Leu Ser Pro Val Ala Arg Glu	2275	2280	2285	
Thr Ser Glu Ile Gly Gly Ser Ser Lys Ala Pro Ser Arg Ser Gly Ser	2290	2295	2300	
Arg Asp Ser Thr Pro Ser Arg Pro Ala Glu Glu Pro Leu Ser Arg Pro	2305	2310	2315	2320
Ile Glu Ser Pro Gly Arg Asn Ser Ile Ser Pro Gly Arg Asn Gly Ile	2325	2330	2335	
Ser Pro Pro Asn Lys Leu Ser Glu Leu Pro Arg Thr Ser Ser Pro Ser	2340	2345	2350	
Thr Ala Ser Thr Lys Ser Ser Gly Ser Gly Lys Met Ser Tyr Thr Ser	2355	2360	2365	
Pro Gly Arg Glu Met Ser Glu Glu Asn Leu Thr Lys Glu Thr Gly Leu	2370	2375	2380	
Ser Lys Asn Ala Ser Ser Ile Pro Arg Ser Glu Ser Ala Ser Lys Gly	2385	2390	2395	2400
Leu Asn Glu Met Asn Asn Gly Asn Gly Ala Asn Lys Lys Val Glu Leu	2405	2410	2415	
Ser Arg Met Ser Ser Thr Lys Ser Ser Gly Ser Glu Ser Asp Arg Ser	2420	2425	2430	
Glu Arg Pro Val Leu Val Arg Glu Ser Thr Phe Ile Lys Glu Ala Pro	2435	2440	2445	
Ser Pro Thr Leu Arg Arg Lys Leu Glu Glu Ser Ala Ser Phe Glu Ser	2450	2455	2460	
Leu Ser Pro Ser Ser Arg Pro Ala Ser Pro Thr Arg Ser Glu Ala Glu	2465	2470	2475	2480
Thr Pro Val Leu Ser Pro Ser Leu Pro Asp Met Ser Leu Ser Thr His	2485	2490	2495	
Ser Ser Val Glu Ala Gly Gly Trp Arg Lys Leu Pro Pro Asn Leu Ser	2500	2505	2510	
Pro Thr Ile Glu Tyr Asn Asp Gly Arg Pro Ala Lys Arg His Asp Ile	2515	2520	2525	
Ala Arg Ser His Ser Glu Ser Pro Ser Arg Leu Pro Ile Asn Arg Ser	2530	2535	2540	
Gly Thr Trp Lys Arg Glu His Ser Lys His Ser Ser Ser Leu Pro Arg	2545	2550	2555	2560
Val Ser Thr Trp Arg Arg Thr Gly Ser Ser Ser Ser Ile Leu Ser Ala	2565	2570	2575	

-continued

Ser Ser Glu Ser Ser Glu Lys Ala Lys Ser Glu Asp Glu Lys His Val
 2580 2585 2590
 Asn Ser Ile Ser Gly Thr Lys Glu Ser Lys Glu Asn Glu Val Ser Ala
 2595 2600 2605
 Lys Gly Thr Trp Arg Lys Ile Lys Glu Asn Glu Phe Ser Pro Thr Asn
 2610 2615 2620
 Ser Thr Ser Glu Thr Val Ser Ser Gly Ala Thr Asn Gly Ala Glu Ser
 2625 2630 2635 2640
 Lys Thr Leu Ile Tyr Glu Met Ala Pro Ala Val Ser Lys Thr Glu Asp
 2645 2650 2655
 Val Trp Val Arg Ile Glu Asp Cys Pro Ile Asn Asn Pro Arg Ser Gly
 2660 2665 2670
 Arg Ser Pro Thr Gly Asn Thr Pro Pro Val Ile Asp Ser Val Ser Glu
 2675 2680 2685
 Lys Ala Asn Pro Asn Ile Lys Asp Ser Lys Asp Asn Glu Ala Lys Glu
 2690 2695 2700
 Asn Val Gly Asn Gly Ser Val Pro Met Arg Thr Val Gly Leu Glu Asn
 2705 2710 2715 2720
 Arg Leu Asn Ser Phe Ile Glu Val Asp Ala Pro Asp Glu Lys Gly Thr
 2725 2730 2735
 Glu Ile Lys Pro Gly Glu Asn Asn Pro Val Pro Val Ser Glu Thr Asn
 2740 2745 2750
 Glu Ser Ser Ile Val Glu Arg Thr Pro Phe Ser Ser Ser Ser Ser
 2755 2760 2765
 Lys His Ser Ser Pro Ser Gly Thr Val Ala Ala Arg Val Thr Pro Phe
 2770 2775 2780
 Asn Tyr Asn Pro Ser Pro Arg Lys Ser Ser Ala Asp Ser Thr Ser Ala
 2785 2790 2795 2800
 Arg Pro Ser Glu Ile Pro Thr Pro Val Asn Asn Asn Thr Lys Lys Arg
 2805 2810 2815
 Asp Ser Lys Thr Asp Ser Thr Glu Ser Ser Gly Thr Glu Ser Pro Lys
 2820 2825 2830
 Arg His Ser Gly Ser Tyr Leu Val Thr Ser Val Lys Arg Gly Arg Met
 2835 2840 2845
 Lys Leu Arg Lys Phe Tyr Val Asn Tyr Asn Cys Tyr Ile Asp Ile Leu
 2850 2855 2860
 Phe Glu Met Lys Leu Lys Thr Glu Lys Phe Cys Lys Val Phe Leu Leu
 2865 2870 2875 2880
 Glu Gly Phe Cys Ser Gly Ser His Ile Tyr Thr Leu Ser Ser Leu Val
 2885 2890 2895
 Leu Phe Trp Glu Ala Leu Leu Met Val Arg Lys Lys Ile Val Lys Pro
 2900 2905 2910
 Ser Met Phe Val Glu Tyr Val Leu His Val Phe Lys Val Ala Pro Ile
 2915 2920 2925
 Pro Thr Ser Phe Asn Tyr Cys Leu Ser Asn Asn Glu His Tyr Arg Lys
 2930 2935 2940
 Ile Tyr Ile Ala Val Ile Asn His Phe Ile Ile Asn Leu Asn Leu His
 2945 2950 2955 2960
 Glu Gly Lys Ile Gly Ile Tyr Ala Lys Lys Asn Val Phe
 2965 2970

(2) INFORMATION FOR SEQ ID NO.8:

(1) SEQUENCE CHARACTERISTICS:

-continued

(A) LENGTH: 486 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: None

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```

Met 1 Pro 5 Gln 10 Leu 15 Asp 20 Ser 25 Gly 30 Gly 35 Gly 40 Ala 45 Gly 50 Gly 55 Asp 60 Asp
Leu 65 Gly 70 Ala 75 Pro 80 Asp 85 Gln 90 Leu 95 Leu 100 Ala 105 Phe 110 Gln 115 Asp 120 Gln 125
Gln 130 Asp 135 Asp 140 Lys 145 Ser 150 Arg 155 Asp 160 Ser 165 Ala 170 Gly 175 Pro 180 Gln 185 Arg 190 Leu 195 Ala
Gln 200 Leu 205 Lys 210 Ser 215 Ser 220 Leu 225 Val 230 Asn 235 Gln 240 Ser 245 Gln 250 Ala 255 Ala 260 Gly 265 Ser
Ala 270 Gly 275 Ile 280 Pro 285 Gly 290 Val 295 Gly 300 Ala 305 Arg 310 Gln 315 Thr 320 Asp 325 Phe 330 Ala 335
Ala 340 Gln 345 Leu 350 Gly 355 Arg 360 Gln 365 His 370 Arg 375 Ala 380 Thr 385 Lys 390 Cys 395 Thr
Ser 400 Gly 405 Met 410 Tyr 415 Lys 420 Gln 425 Thr 430 Val 435 Tyr 440 Ser 445 Ala 450 Phe 455 Asn 460 Leu 465 Met
His 470 Tyr 475 Pro 480 Pro 485 Ser 490 Gly 495 Ala 500 Gly 505 Gln 510 His 515 Pro 520 Gln 525 Pro 530
Pro 535 Leu 540 His 545 Lys 550 Ala 555 Asn 560 Gln 565 Pro 570 Pro 575 His 580 Gly 585 Val 590 Pro 595 Gln 600 Ser
Leu 605 Tyr 610 Gln 615 His 620 Phe 625 Asn 630 Ser 635 Pro 640 His 645 Pro 650 Thr 655 Pro 660 Ala 665 Pro 670 Ala 675
Ile 680 Ser 685 Gln 690 Lys 695 Gln 700 Val 705 His 710 Arg 715 Pro 720 Leu 725 Gln 730 Thr 735 Pro 740 Asp 745 Leu 750 Ser
Gly 755 Phe 760 Tyr 765 Ser 770 Leu 775 Thr 780 Ser 785 Gly 790 Ser 795 Met 800 Gly 805 Gln 810 Leu 815 Pro 820 His 825 Thr
Val 830 Ser 835 Trp 840 Pro 845 Ser 850 Pro 855 Pro 860 Leu 865 Tyr 870 Pro 875 Leu 880 Ser 885 Pro 890 Ser 895 Cys 900 Gly
Tyr 905 Arg 910 Gln 915 His 920 Phe 925 Pro 930 Ala 935 Pro 940 Thr 945 Ala 950 Ala 955 Pro 960 Gly 965 Ala 970 Pro 975 Tyr
Pro 980 Arg 985 Phe 990 Thr 995 His 1000 Pro 1005 Ser 1010 Leu 1015 Met 1020 Leu 1025 Gly 1030 Ser 1035 Gly 1040 Val 1045 Pro 1050 Gly
His 1055 Pro 1060 Ala 1065 Ala 1070 Ile 1075 Pro 1080 His 1085 Pro 1090 Ala 1095 Ile 1100 Val 1105 Pro 1110 Pro 1115 Ser 1120 Gly 1125 Lys
Gln 1130 Gln 1135 Leu 1140 Gln 1145 Pro 1150 Phe 1155 Asp 1160 Arg 1165 Asn 1170 Leu 1175 Lys 1180 Thr 1185 Gln 1190 Ala 1195 Gln 1200 Ser
Lys 1205 Ala 1210 Gln 1215 Lys 1220 Gln 1225 Ala 1230 Lys 1235 Lys 1240 Pro 1245 Thr 1250 Ile 1255 Lys 1260 Lys 1265 Pro 1270 Leu 1275 Asn
Ala 1280 Phe 1285 Met 1290 Leu 1295 Tyr 1300 Met 1305 Lys 1310 Gln 1315 Met 1320 Arg 1325 Ala 1330 Lys 1335 Val 1340 Ile 1345 Ala 1350 Gln
Cys 1355 Thr 1360 Leu 1365 Lys 1370 Gln 1375 Ser 1380 Ala 1385 Ala 1390 Ile 1395 Asn 1400 Gln 1405 Ile 1410 Leu 1415 Gly 1420 Arg 1425 Arg
Trp 1430 His 1435 Ala 1440 Leu 1445 Ser 1450 Arg 1455 Gln 1460 Gln 1465 Gln 1470 Ala 1475 Lys 1480 Tyr 1485 Tyr 1490 Gln 1495 Leu 1500 Ala
Arg 1505 Lys 1510 Gln 1515 Arg 1520 Gln 1525 Leu 1530 His 1535 Met 1540 Gln 1545 Leu 1550 Tyr 1555 Pro 1560 Gly 1565 Trp 1570 Ser 1575 Ala
Arg 1580 Asp 1585 Asn 1590 Tyr 1595 Gly 1600 Lys 1605 Lys 1610 Arg 1615 Arg 1620 Ser 1625 Arg 1630 Gln 1635 Lys 1640 His 1645 Gln

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Glu Ser Thr Thr Gly Gly Lys Arg Asn Ala Phe Gly Thr Tyr Pro Glu
 385 390 395 400
 Lys Ala Ala Ala Pro Ala Pro Phe Leu Pro Met Thr Val Leu Ala Ala
 405 410 415
 Pro Gly Pro Gln Leu Pro Arg Thr His Pro His Thr Ile Cys Cys Pro
 420 425 430
 Ala Ser Pro Gln Asn Cys Leu Leu Ala Leu Arg Ser Arg His Leu His
 435 440 445
 Pro Gln Val Ser Pro Leu Leu Ser Ala Ser Gln Pro Gln Gly Pro His
 450 455 460
 Arg Pro Pro Ala Ala Pro Cys Arg Ala His Arg Tyr Ser Asn Arg Asn
 465 470 475 480
 Leu Arg Asp Arg Trp Pro
 485

(2) INFORMATION FOR SEQ ID NO:9:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 511 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(11) MOLECULE TYPE: None

(12) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Met Pro Gln Leu Asp Ser Gly Gly Gly Gly Ala Gly Gly Gly Asp Asp
 1 5 10 15
 Leu Gly Ala Pro Asp Gln Leu Leu Ala Phe Gln Asp Gln Gly Gln Gln
 20 25 30
 Gln Asp Asp Lys Ser Arg Asp Ser Ala Gly Pro Gln Arg Asp Leu Ala
 35 40 45
 Gln Leu Lys Ser Ser Leu Val Asn Gln Ser Gln Gly Ala Ala Gly Ser
 50 55 60
 Ala Gly Ile Pro Gly Val Pro Gly Ala Gly Ala Gly Ala Arg Gly Gln
 65 70 75 80
 Ala Gln Ala Leu Gly Arg Gln His Arg Ala Gln Arg Leu Phe Pro Asp
 85 90 95
 Lys Leu Pro Gln Pro Leu Gln Asp Gly Leu Lys Ala Pro Gln Cys Thr
 100 105 110
 Ser Gly Met Tyr Lys Gln Thr Val Tyr Ser Ala Phe Asn Leu Leu Met
 115 120 125
 His Tyr Pro Pro Pro Ser Gly Ala Gly Gln His Pro Gln Pro Gln Pro
 130 135 140
 Pro Leu His Lys Ala Asn Gln Pro Pro His Gly Val Pro Gln Leu Ser
 145 150 155 160
 Leu Tyr Gln His Phe Asn Ser Pro His Pro Thr Pro Ala Pro Ala Asp
 165 170 175
 Ile Ser Gln Lys Gln Val His Arg Pro Leu Gln Thr Pro Asp Leu Ser
 180 185 190
 Gly Phe Tyr Ser Leu Thr Ser Gly Ser Met Gly Gln Leu Pro His Thr
 195 200 205
 Val Ser Trp Pro Ser Pro Pro Leu Tyr Pro Leu Ser Pro Ser Cys Gly
 210 215 220
 Tyr Arg Gln His Phe Pro Ala Pro Thr Ala Ala Pro Gly Ala Pro Tyr
 225 230 235 240

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Pro Arg Phe Thr His Pro Ser Leu Met Leu Gly Ser Gly Val Pro Gly
      245      250      255
His Pro Ala Ala Ile Pro His Pro Ala Ile Val Pro Pro Ser Gly Lys
      260      265      270
Gln Glu Leu Gln Pro Phe Asp Arg Asn Leu Lys Thr Gln Ala Glu Ser
      275      280      285
Lys Ala Glu Lys Gln Ala Lys Lys Pro Thr Ile Lys Lys Pro Leu Asn
      290      295      300
Ala Phe Met Leu Tyr Met Lys Glu Met Arg Ala Lys Val Ile Ala Glu
      305      310      315
Cys Thr Leu Lys Glu Ser Ala Ala Ile Asn Gln Ile Leu Gly Arg Arg
      325      330      335
Trp His Ala Leu Ser Arg Glu Glu Gln Ala Lys Tyr Tyr Gln Leu Ala
      340      345      350
Arg Lys Glu Arg Gln Leu His Met Gln Leu Tyr Pro Gly Trp Ser Ala
      355      360      365
Arg Asp Asn Tyr Gly Lys Lys Lys Arg Arg Ser Arg Glu Lys His Gln
      370      375      380
Glu Ser Thr Thr Asp Pro Gly Ser Pro Lys Lys Cys Arg Ala Arg Phe
      385      390      395
Gly Leu Asn Gln Gln Thr Asp Tip Cys Gly Pro Cys Arg Arg Lys Lys
      405      410      415
Lys Cys Ile Arg Tyr Leu Pro Gly Gln Gly Arg Cys Pro Ser Pro Val
      420      425      430
Pro Ser Asp Asp Ser Ala Leu Gly Cys Pro Gly Ser Pro Ala Pro Gln
      435      440      445
Asp Ser Pro Ser Tyr His Leu Leu Pro Arg Phe Pro Thr Glu Leu Leu
      450      455      460
Thr Ser Pro Ala Glu Pro Ala Pro Thr Ser Pro Gly Leu Ser Thr Ala
      465      470      475
Leu Ser Leu Pro Thr Pro Gly Pro Pro Gln Ala Pro Arg Ser Thr Leu
      485      490      495
Gln Ser Thr Gln Val Gln Gln Gln Glu Ser Gln Arg Gln Val Ala
      500      505      510

```

(2) INFORMATION FOR SEQ ID NO:10:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: None

(11) SEQUENCE DESCRIPTION: SEQ ID NO:10

```

Ser Tyr Leu Asp Ser Gly Ile His Ser Gly Ala Thr Thr Thr Ala Pro
1      5      10      15
Ser Leu Ser Gly
      20

```

(2) INFORMATION FOR SEQ ID NO:11:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: None

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Ser Tyr Leu Gly Asp Ser Gly Ile His Ser Gly Ala Val Thr Gln Val
 1 5 10 15
 Pro Ser Leu Ser Gly
 20

We claim:

1. A method of identifying candidate drugs for use in Familial Adenomatous Polyposis (FAP) patients, patients with Adenomatous Polyposis Coli (APC) or β -catenin mutations, or patients with increased risk of developing colorectal cancer, comprising the steps of:

contacting a cell having no wild-type APC or a mutant β -catenin with a test compound, wherein said cell comprises a TCF-responsive reporter gene;

measuring transcription of the Tcf-responsive reporter gene in said cell, wherein a test compound which inhibits the transcription of the reporter gene in said cell is a candidate drug for cancer therapy.

2. The method of claim 1 wherein the cell produces an APC protein defective in β -catenin binding or regulation.

3. The method of claim 1 wherein the cell produces a β -catenin protein which is super-active, or which is defective in APC binding or resistant to APC regulation.

4. The method of claim 1 wherein the cell produces no detectable APC protein.

5. A method of identifying candidate drugs for use in FAP patients, patients with APC or β -catenin mutations, or patients with increased risk of developing colorectal cancer, comprising the steps of:

contacting a Tcf-responsive reporter gene with a test compound under conditions in which the reporter gene is transcribed in the absence of the test compound; and

measuring transcription of the Tcf-responsive reporter gene; wherein a test compound which inhibits said transcription is a candidate drug for cancer therapy.

6. The method of claim 5 wherein the step of contacting is performed in the presence of a lysate of a cell which has no wild-type APC.

7. The method of claim 5 wherein the step of contacting is performed in the presence of a lysate of a cell which has a mutant β -catenin defective in APC binding or resistant to APC regulation or which is super-active.

8. The method of claim 6 wherein the cell produces an APC protein defective in β -catenin binding or regulation.

9. A method of identifying candidate drugs for use in FAP patients or patients with increased risk of developing colorectal cancer, comprising the steps of:

contacting a test compound with β -catenin and Tcf-4 under conditions in which β -catenin and Tcf-4 bind to each other; and

determining whether the test compound inhibits the binding of β -catenin and Tcf-4, a test compound which inhibits the binding being a candidate for cancer therapy or prophylaxis.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 5,851,775
DATED : December 22, 1998
INVENTOR(S) : Barker et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 3, line 17, change "FIG. 1" to --FIGS. 1A, 1B and 1C--.

Column 3, line 29, change "FIG. 2" to --FIGS. 2A, 2B and 2C--.

Column 3, line 43, change "FIGS. 3A, 3B" to --FIGS. 3A, 3B and 3C--.

Column 4, line 44, change "FIGS. 7A, 7B and 7C" to --FIGS. 7A and 7B--.

Column 5, line 6, change "B" to --8B--.

Signed and Sealed this
Thirteenth Day of July, 1999

Attest:



Q. TODD DICKINSON

Attesting Officer

Acting Commissioner of Patents and Trademarks

Tyrosine phosphorylation of the MUC1 breast cancer membrane protein: Cytokine receptor-like molecules

Sheila Zrihan-Licht, Amos Baruch, Orna Elroy-Stein, Iafa Keydar, Daniel H. Wreschner*

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Received 12 September 1994; revised version received 27 October 1994

Abstract Phosphorylation on tyrosine residues is a key step in signal transduction pathways mediated by membrane proteins. Although it is known that human breast cancer tissue expresses at least 2 MUC1 type 1 membrane proteins (a polymorphic high molecular weight MUC1 glycoprotein array) their function in the development of human breast cancer has remained elusive. Here it is shown that these MUC1 proteins are extensively phosphorylated, that phosphorylation occurs primarily on tyrosine residues and that following phosphorylation the MUC1 proteins may potentially interact with SH2 domain-containing proteins and thereby initiate a signal transduction cascade. As with cytokine receptors, the MUC1 protein complex whose formation is mediated by two cytoplasmically located MUC1 cysteine residues. Furthermore, the MUC1/Y protein demonstrates sequence similarity with sequences present in cytokine receptors that are known to be involved in ligand binding. Our results demonstrate that the two MUC1 isoforms are both likely to function in signal transduction pathways and to be intimately linked to the oncogenic process and suggest that the MUC1/Y protein may act in a similar fashion to cytokine receptors.

Key words: Breast cancer; Tyrosine phosphorylation; Receptor; MUC1

1. Introduction

Protein products of the MUC1 gene are expressed at high levels in adenocarcinomas and especially in human breast cancer tissue [1–7] and disease status in breast cancer patients is routinely assessed by monitoring the serum levels of circulating MUC1 proteins (variously referred to as episialin, H23Ag, ETA – epithelial tumor antigen, PEM – polymorphic epithelial mucin, EMA – epithelial membrane antigen, CA15-3, MCA – mammary carcinoma antigen, etc.). Molecular studies, including cDNA and gene cloning [8–14], have elucidated many properties of the MUC1 proteins. One of the MUC1 gene products is a polymorphic type 1 transmembrane molecule that consists of a large extracellular domain, a transmembrane domain and a 72 amino acid cytoplasmic tail (Fig. 1F, upper molecule). The genetic polymorphism derives from a tandem array of variable numbers of a highly conserved 20 amino acid repeat motif present within the extracellular domain. Soon after translation and prior to its translocation to the cell surface, this MUC1 protein (designated MUC1/REP) undergoes proteolytic cleavage in a region that is located 45 to 60 amino acids N-terminal to the transmembrane domain [15]. The two resulting protein molecules form a tight heterodimeric complex that is composed of the large extracellular domain linked by non-covalent, SDS sensitive bonds to the much smaller (20–30 kDa) protein molecule containing the cytoplasmic and transmembrane domains [15]. Expression of the MUC1/REP protein in cell transfectants reduces cellular aggregation that is mediated by the highly glycosylated tandem repeat domain [16].

An additional novel MUC1 protein (designated MUC1/Y) has been recently characterized [8] that is devoid of the hallmark feature of MUC1, the tandem repeat array, yet retains the MUC1 N-terminal, transmembrane and cytoplasmic domains (Fig. 1F, lower molecule). The MUC1/Y protein is generated by a splicing mechanism that utilizes perfect alternative splice

donor and splice acceptor sites located upstream and downstream to the tandem repeat array. Previous work demonstrated that the mature MUC1/Y protein has a molecular mass of between 42–45 kDa indicating that, in contrast to the cleaved MUC1/REP, it does not undergo proteolytic cleavage and is therefore continuous from its N-terminal extracellular domain through to its C-terminal cytoplasmic domain. In addition to the mature 42–45 kDa MUC1/Y protein, a precursor 33 kDa MUC1/Y protein that subsequently undergoes post-translational glycosylation modifications was also identified [8]. Significantly, both the MUC1/REP and MUC1/Y isoforms are highly expressed in human breast cancer tissue [1–8].

As the MUC1/REP and the novel MUC1/Y proteins are anchored at the cell surface and contain extracellular and common transmembrane and cytoplasmic domains they may both be involved in signal transduction processes.

Membrane proteins participating in signal transduction processes are in many cases modified by phosphorylation. It has not been known whether the MUC1 proteins are at all phosphorylated – indeed the MUC1 cytoplasmic domain does not contain any conserved sequence motifs known to exist in the catalytic domains of kinases in general or tyrosine kinases in particular, and is thus devoid of endogenous kinase activity and cannot undergo autophosphorylation. Similarly, cytokine receptors are also devoid of intrinsic kinase activity but are in many instances transphosphorylated on tyrosine residues by cytoplasmic tyrosine kinases [17–21]. It is shown here, for the first time, that the MUC1 proteins are phosphorylated on tyrosine residues and that following phosphorylation they have the potential to interact with SH2 domain containing proteins and thereby initiate a signal transduction cascade. Furthermore we show that, as with cytokine receptors, the MUC1/Y isoform participates in a cell-surface heteromeric complex. Interestingly, the MUC1/Y protein demonstrates sequence similarity with sequences present in cytokine receptors that are known to be involved in ligand binding. Our results demonstrate that the two MUC1 isoforms are both likely to function in signal

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transduction pathways and to be intimately linked to the oncogenic process and suggest that the MUC1Y protein may act in a similar fashion to cytokine receptors.

2. Materials and methods

2.1. Cells

Stable transfectants were generated by co-transfecting an expression plasmid harboring either MUC1/REP or MUC1Y cDNA with the neomycin plasmid (pSV2 neo) selection marker into HBL100 human mammary epithelial cells (MUC1/REP transfectants) or into 3T3 ras transformed fibroblasts (MUC1Y transfectants). These cells were chosen as they showed the highest expression levels of the respective MUC1 isoforms. Transient transfectants were prepared using the T7/ovary-lonovirus/vaccinia system (described below) and the monkey epithelial cell line, BSC-1.

2.2. cDNA constructs

The generation of expression vectors harboring either the full-length transmembrane MUC1/REP or the novel MUC1Y cDNA and driven by the HMG coenzyme A reductase promoter (expression vector pCL4-12) has been previously described [8].

2.3. Transient expression of the novel MUC1Y protein in the T7/EMC1 vaccinia system

Transient expression of the novel MUC1Y protein synthesized in a state as close as possible to the naturally occurring MUC1Y protein, was accomplished using the T7/EMC1/vaccinia hybrid expression system [9]. This system utilizes the bacteriophage T7 RNA polymerase which is encoded by a recombinant vaccinia virus to transcribe genes that are regulated by the T7 promoter in the cytoplasm of infected mammalian cells. The MUC1Y cDNA was inserted into the pTMI1 vector under the control of the T7 promoter and EMCV leader, and introduced into tissue cultured BSC-1 epithelial cells together with recombinant vaccinia virus which expresses T7 RNA polymerase [8].

2.4. Western blot analyses

Cell lysates were prepared by adding lysis buffer (50 mM NaCl, 20 mM Tris-HCl, pH 7.4, 100 μ M leupeptin and 0.5% Nonidet P-40) to cell pellets, followed by vortex mixing and sonication (3 times 10 second bursts using a Branson sonicator). Cell debris was removed by centrifugation at 10,000 rpm for 10 min. All procedures were performed at 4°C or on ice. Protein samples were denatured by boiling in SDS buffer containing mercaptoethanol and analyzed on SDS/polyacrylamide gels. The gel was electrophoretically transferred for 3 h at 1 A to nitrocellulose filters that were then blocked in PBS containing 5% skimmed milk followed by incubation with the primary antibody. The filters were washed in PBS and then incubated with a secondary anti-rabbit (or anti-mouse antibody) conjugated to horseradish peroxidase followed by ECL (Amersham) detection.

2.5. Antibodies

Western blot analyses were performed with a polyclonal antibody (a kind gift from Dr. Sandra Gendler) directed against the oligopeptide SLSTYNFAVAATSANL (amino acids 499 to 515, see [8] for amino acid numbering) which represents the C-terminal region of the MUC1 cytoplasmic domain. The polyclonal antibody was preabsorbed by adsorption against glutaraldehyde insolubilized human serum.

2.6. Metabolic labelling of cells with phosphate

Cells were incubated overnight in low phosphate medium supplemented with 10% fetal calf serum that had been dialyzed against saline. The following day radioactive carrier-free inorganic phosphate was added to the cells and incubation continued for another 8 h. Thirty minutes prior to harvesting, the cells were treated with the tyrosine phosphatase inhibitors, sodium vanadate (200 μ M) and hydrogen peroxide (200 μ M).

2.7. Immunoprecipitations

Cell lysates prepared as described above were added to protein-A-sepharose-antibody complex and incubated for 2 h at 4°C. The immunocomplex was washed 3 times with cell lysis buffer and 2 x SDS sample buffer was added.

3. Results and discussion

To investigate whether MUC1 is transphosphorylated, stable transfectants expressing either the MUC1/REP protein or the novel MUC1Y protein were generated. Immunoblotting experiments with antibodies directed against the MUC1 cytoplasmic domain confirmed MUC1/REP (20-30 kDa immunoreactive proteins) and MUC1Y (42-45 kDa immunoreactive proteins) expression in the respective transfectants (Fig. 1A). MUC1 expressing transfectants were incubated with radioactively labelled inorganic phosphate in the presence of the tyrosine phosphatase inhibitors, hydrogen peroxide and sodium vanadate [22,23], cell lysates were then prepared and subjected to immunoprecipitation with anticytoplasmic domain antibodies. The specifically immunoprecipitated proteins migrating with molecular masses of 20-30 kDa for the MUC1/REP protein (Fig. 1B, lane 2) and 42-45 kDa for the MUC1Y protein (Fig. 1B, lane 6) were highly labelled, indicating that the MUC1 proteins had undergone extensive phosphorylation. Similarly the MUC1 proteins were also found to be phosphorylated in non-transfected human T47D breast cancer cells (data not shown). Due however to the considerably lower level of expression as compared to that in the MUC1 transfectants, the signal of the phosphorylated MUC1 proteins in the T47D cells was correspondingly lower and further work thus conducted with the MUC1 transfectants.

The effect of the tyrosine phosphatase inhibitors on the levels of MUC1 phosphorylation was next investigated. In the absence of these inhibitors, MUC1 phosphorylation demonstrated a low yet significant level of phosphorylation that in their presence was markedly enhanced (Fig. 1C,D) suggesting that phosphorylation of the MUC1 proteins occurs predominantly on tyrosine residues. Consistent with this, nonspecifically precipitated labelled proteins (Fig. 1C, open arrow at left of figure) showed no differential enhancement of phosphorylation following treatment of cells with the tyrosine phosphatase inhibitors.

A phosphoamino acid analysis performed on the labelled phosphorylated MUC1 proteins showed that phosphorylation had indeed primarily occurred on tyrosine residues (70-90% in different experiments), with much reduced levels of phosphoserine and undetectable levels of threonine phosphorylation (Fig. 1E (a)). This pattern of tyrosine phosphorylation was observed both for the MUC1/REP and MUC1Y proteins. Further confirmation for tyrosine phosphorylation of the MUC1 proteins was obtained by probing immunoblots of immunoprecipitated MUC1 proteins with antiphosphotyrosine antibodies. This analysis (Fig. 1E (b)) clearly showed that the MUC1Y protein is readily detected by antiphosphotyrosine antibodies following treatment of cells with tyrosine phosphatase inhibitors.

Three independent lines of evidence thus support the finding that the MUC1 proteins are phosphorylated on tyrosine residues: (i) increased levels of MUC1 phosphorylation following treatment of cells with tyrosine phosphatase inhibitors, (ii) a phosphoamino acid analysis of the MUC1 proteins, and (iii) reactivity of phosphorylated MUC1 proteins with antiphosphotyrosine antibodies.

Interestingly, tyrosine residues are distributed in a markedly biased fashion within the MUC1 proteins - 7 out of 72 of the amino acids comprising the MUC1 cytoplasmic domain are

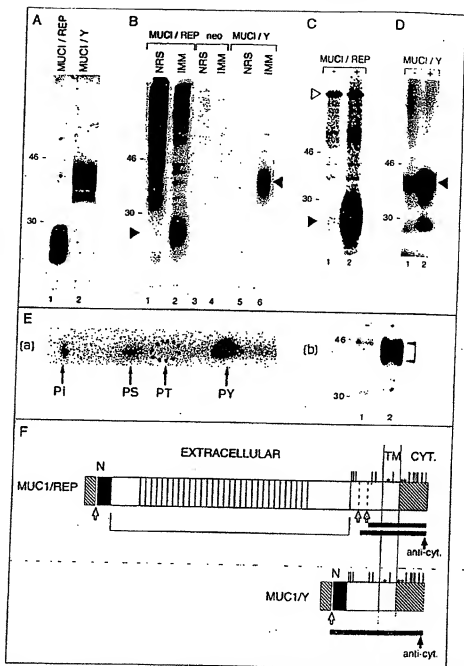


Fig. 1. Phosphorylation of the MUC1 proteins on phosphotyrosine residues. (A) MUC1/REP and MUC1/Y expression in stable transfectants. Lysates were prepared from the MUC1/REP (lane 1) and MUC1/Y (lane 2) and the proteins resolved on SDS-polyacrylamide (10%) gels, transferred to nitrocellulose and probed with polyclonal antisera directed against the MUC1 cytoplasmic domain, as described in section 2. Molecular size standards (in kilodaltons) are shown at left. (B) Phosphorylation of the MUC1 proteins. The MUC1/REP transfectants (lanes 1 and 2, MUC1/REP), control neomycin transduced ras 3T3 fibroblasts (lanes 3 and 4, neo) and MUC1/Y ras 3T3 fibroblast transfectants (lanes 5 and 6, MUC1/Y) were labelled with radioactive carrier-free inorganic phosphate (section 2). The cells were then harvested, briefly washed with phosphate-buffered saline and cell lysates prepared that were then subjected to immunoprecipitation with either preimmune rabbit serum (NRS, lanes 1, 3 and 5) or anticytoplasmic domain antisera (IMM, lanes 2, 4 and 6). Precipitated proteins were resolved on SDS-polyacrylamide (10%) gels visualized by autoradiography. (C) and (D) Effect of tyrosine phosphatase inhibitors on MUC1/REP (C) and MUC1/Y (D) phosphorylation.

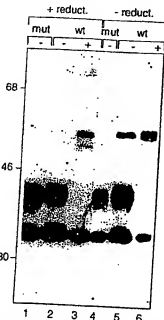


Fig. 2. The MUC1Y protein appears in a cell surface complex, the formation of which is mediated via MUC1 cytoplasmic domain cysteine residues. Cell lysates were prepared from monkey BSC-1 cells infected with recombinant vaccinia virus coding for T7 RNA polymerase and transfected with either wild type MUC1Y expression vector harboring cDNA coding for mutant MUC1Y protein (mut, lanes 1 and 4) in which the Cys-Gln (CQC) sequence had been mutated to Gly-Gln-Gly (see Fig. 1F for the location of the CQC sequence). The cell lysate proteins were resolved on SDS polyacrylamide (10%) gels under non-reducing (- reduct., lanes 4-6) or reducing (+ reduct., lanes 1-3) conditions, transferred to nitrocellulose and probed with polyclonal antisera directed against the MUC1 cytoplasmic domain. Proteins resolved in lanes 3 and 6 were derived from cells that prior to harvesting had been treated for 15 min with 15 mM EDAC crosslinking agent (obtained from Sigma). Detection of bound antibodies was performed as described in Fig. 1A.

Fig. 1 (continued).

MUC1REP or MUC1Y transfectants were labelled with radioactive carrier-free inorganic phosphate as described above and either treated with sodium vanadate and hydrogen peroxide (lane 2, +) or not treated (lane 1, -). Proteins immunoprecipitated with anticytoplasmic domain antibodies were visualized as above. Non-specifically immunoprecipitated proteins are indicated in (C) by the open arrow; the closed arrows indicate the specifically immunoprecipitated MUC1 proteins. (E) Phosphoamino acid analysis. The immunoprecipitated phosphorylated MUC1 proteins (from lane 2 in (B)) were isolated from SDS-acrylamide (10%) gel and hydrolyzed in 6M HCl at 110°C for 1 h. Labelled phosphoaminoacids (with added 10% of internal phosphoamino acid markers) were analyzed by thin-layer high voltage electrophoresis followed by Phosphorimager analysis. The position of migration of phosphoserine, phosphothreonine and phosphotyrosine are indicated by PS, PT and PY respectively and inorganic phosphate by P_i. Phosphoamino acid analyses performed on the phosphorylated MUC1 cleavage products (lanes 1 and 2 in (C)) and the phosphorylated MUC1Y proteins (lanes 1 and 2 in (D)) gave similar results (data not shown). (b) Reactivity of MUC1Y protein with anti-phosphotyrosine antibodies. Transfected and protein immunoprecipitated with anticytoplasmic domain antibodies were resolved on SDS-polyacrylamide (10%) gels followed by Phosphorimager analysis. The arrowed region indicates the tyrosine phosphorylated MUC1Y protein. (f) Scheme depicting the repeat array containing MUC1 protein (upper molecule) and the novel MUC1Y protein (lower molecule). The location of tyrosine and cysteine residues are indicated above the rectangles by vertical lines and asterisks, respectively. Both MUC1 proteins contain a hydrophobic N-terminal signal sequence (shaded box at left of figure) that is co-translationally cleaved (arrow at left of figure). This cleavage is followed by the tandem repeat array (upper molecule) that is illustrated by the block of closely spaced vertical lines. The highly hydrophobic 28 amino acid stretch constituting the transmembrane domain (TM) is shown at the C-terminal end of both MUC1 proteins, followed by the cytoplasmic domain (CYT). The region comprising the proteolytic cleavage site [15] of the repeat array containing MUC1 protein (upper molecule) is indicated by 2 vertical dotted arrows just N-terminal to the transmembrane domain. The regions recognized by the anti-cytoplasmic domain antibodies are indicated.

tyrosine residues (Fig. 1F). The transmembrane domain contains one tyrosine residue and a further 5 tyrosine residues appear within the 92 amino acids N-terminally adjacent to the transmembrane domain – no additional tyrosine residues appear within the MUC1 proteins. The MUC1 amino acid sequence also reveals a marked similarity between tyrosine containing sequences located within the MUC1 cytoplasmic domain and phosphotyrosine containing peptide sequences that are postulated to specifically interact with SH2 domain containing proteins [26]. It should be emphasized that these sites represent only *presumptive* docking sites for SH2 domain containing proteins; it is nonetheless striking that the 72 amino acid MUC1 cytoplasmic domain contains no less than 3 such possible sites. For example, the most preferred sequence for interaction with phospholipase C γ 1 is pTyr-Val-Iso-Pro (pYVIF) and a very similar sequence pTyr-Val-Pro-Pro (pYVPP) appears in the cytoplasmic domain of the MUC1 protein. Additionally the sequence pTyr-Glu-Glu-Val (pYEEV) which is identical to a sequence that appears within the mouse MUC1 cytoplasmic domain, has been shown to be one of the most preferred sequences for interaction with a number of SH2 domain containing cytoplasmic tyrosine kinases [26] and a potential GRB-2 binding site (pYXNQ) also appears in the MUC1 cytoplasmic domain. That the MUC1 cytoplasmic domain has the potential to interact with SH2 domain containing proteins has been experimentally demonstrated by the binding of in-vitro tyrosine phosphorylated MUC1 cytoplasmic domain to the src SH2 domain, the SH2 domain derived from the N-terminal part of phospholipase C and to the GRB-2 protein (data not shown); no binding was observed to the SH2 domain derived from the C-terminal portion of p85 phosphatidylinositol (PI) 3' kinase. One should bear in mind that in-vitro tyrosine phosphorylated MUC1 cytoplasmic domain may not faithfully reflect the tyrosine phosphorylation state of this protein within the cell; experiments investigating the actual association of the MUC1 protein with SH2 domain second messenger proteins in vivo are presently being conducted. Nonetheless, the analyses described above do indicate that the tyrosine phosphorylated MUC1 protein certainly has the potential to participate in such interactions.

human IL-7 receptor shows 44% homology with MUC1 extracellular domain sequences over a stretch of 27 amino acids that span the ligand binding site - if one also includes in this homology comparison valine to leucine and valine to methionine substitutions, the extent of homology increases to 55%. Significantly this homology maps in close proximity to the region where proteolytic cleavage occurs in the MUC1/REP protein, suggesting that integrity of this site in the MUC1/REP protein may be of prime importance for both ligand binding and signal transmission and that different mechanisms may be responsible for activation of the two MUC1 isoforms. The MUC1/REP protein, however, contains neither the conserved extracellular domain cysteine residues nor the Trp-Ser-XXX-Trp-Ser motif that are characteristic of many cytokine receptors [20] - it is notable that this latter motif appears in the human growth hormone receptor as Tyr-Gly-Glu-Phe-Ser and not as the canonical WSXWS. It is therefore interesting that a Phe-Ser-XXX-XXX-Ser motif (Phe-Ser-Ala-Glu-Ser) does appear in the MUC1 sequence just N-terminal to the transmembrane domain, at an identical location to the WSXWS motif, seen in cytokine receptors.

Taken together, these data demonstrate that the MUC1 proteins participate in signal transduction and that the MUC1/REP protein may act as a cytokine receptor-like molecule.

It has been shown here for the first time that the MUC1 proteins are extensively phosphorylated, that phosphorylation occurs on tyrosine residues and that following phosphorylation the MUC1 proteins may potentially interact with SH2 domain containing proteins and thereby initiate a signal transduction cascade. As with cytokine receptors [17], the MUC1/REP protein does not harbor intrinsic tyrosine kinase activity yet is tyrosine phosphorylated, and participates in a cell-surface heteromeric complex - furthermore, the MUC1/REP protein demonstrates sequence similarity with sequences present in cytokine receptors that are known to be involved in ligand binding. Indeed, the striking enhancement of MUC1 phosphorylation (Fig. 1C,D) elicited by the tyrosine phosphatase inhibitors suggests the existence of regulatory mechanisms, such as ligand binding to the MUC1 extracellular domains and/or cellular redox potential changes [37] that may control MUC1 tyrosine phosphorylation levels by activating cytoplasmic kinases which subsequently transphosphorylate the MUC1 proteins. These features suggest that the MUC1/REP protein may act in a similar fashion to cytokine receptors and that following binding of an yet unidentified ligand, undergo transphosphorylation mediated by cytoplasmic tyrosine kinases such as the Janus kinases [17,30].

Notwithstanding the fact that the two MUC1 isoforms have identical cytoplasmic domains, are phosphorylated on tyrosine residues and are both likely to participate in signal transduction processes, the difference in their extracellular domain structure and the cleavage of the MUC1/REP form as opposed to the integrity of MUC1/REP all argue against identical functions as well as activating mechanisms for the two isoforms.

We have previously shown that both the MUC1/REP and MUC1 proteins are highly expressed in human breast cancer tissue [8]. The elucidation of mechanisms that activate the cell-surface located MUC1 proteins, shown here to be intimately linked to signal transduction and oncogenic processes, may lead to new modalities for the treatment of human breast cancer.

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